INFECTION AND IMMUNITY, Sept. 1998, p. 4507–4510 Vol. 66, No. 9
0019-9567/98/$04.00+0

Antibody Responses in Humans against Coli Surface Antigen 6 of Enterotoxigenic *Escherichia coli*

ANNA HELANDER,[1]* CHRISTINE WENNERÅS,[1] FIRDAUSI QADRI,[2] AND ANN-MARI SVENNERHOLM[1]

Department of Medical Microbiology and Immunology, Göteborg University, Göteborg, Sweden,[1] and the International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh[2]

Received 12 November 1997/Returned for modification 21 January 1998/Accepted 17 June 1998

Enterotoxigenic *Escherichia coli* (ETEC) strains expressing only coli surface antigen 6 (CS6) have previously been isolated from patients with diarrhea, but the immunogenicity of CS6 has not been established in humans. We have detected CS6-specific immunoglobulin A responses in the feces and blood of patients convalescing from natural ETEC disease and of volunteers given an oral ETEC vaccine.

Enterotoxigenic *Escherichia coli* (ETEC) is a common cause of diarrhea in developing countries, affecting children and travelers in these areas. The bacteria attach to the intestinal mucosa by means of colonization factors (CFs) and secrete diarrheagenic enterotoxins (heat-labile toxin [LT] and/or heat-stable toxin [ST]) (5). Among the most studied CFs are CF antigen I (CFA/I) and coli surface antigens 1 to 6 (CS1 to CS6) (5). A large proportion of ETEC strains express the nonfimbrial CS6 alone or in combination with either the fimbrial CS4 or the fibrillar CS5 (5). Whereas CFA/I and CS1 to CS5 have been reported to induce immune responses locally in the intestine as well as in serum after ETEC disease or oral vaccination (1, 16–18), studies of the immunogenicity of CS6 in humans have not been reported. However, there is some evidence that CS6 may be an important virulence factor as well as a protective antigen. Thus, CS6-only strains have been shown to colonize the small intestine and to induce protective immunity in rabbits (20). Furthermore, ETEC strains expressing only CS6 have been isolated as the only pathogen from patients with diarrhea (2, 21), and we have recently shown that CS6-expressing ETEC binds to isolated human enterocytes in vitro (7).

The aim of this study was to establish methods for the determination of CS6-specific antibodies and to investigate whether CS6 can induce local and/or systemic immune responses in humans after infection or vaccination. This was done by analyzing, by different enzyme-linked immunosorbent assays (ELISAs) and immunoblotting, plasma or serum and fecal specimens obtained from patients convalescing from diarrhea caused by CF-positive ETEC as well as from healthy volunteers vaccinated with an oral inactivated ETEC vaccine. The patient group consisted of 10 Bangladeshi adults with acute watery diarrhea due to ETEC infection who were admitted to the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh. Six of the patients were infected with CS5- and CS6-positive ($CS5^+$ $CS6^+$) strains. Four patients, three of whom were infected with CFA/I^+ ETEC strains and one of whom was infected with a $CS1^+$ $CS3^+$ strain, were included as controls. After signed informed consent was obtained from the patients, fecal samples were collected on the day of admission (day 0; acute-phase sample) and 9 days later (day 9; convalescent-phase sample); the day 0 specimens were cultured on CFA agar with and without bile salts (4, 13). Four to five *E. coli* colonies isolated from each patient were assayed for CF expression by slide agglutination (11) or in a dot blot test (8), using monoclonal antibodies (MAbs) specific for CFA/I (11), CS1 (12), CS2 (12), CS3 (12), CS4 and CS5 (20a), and CS6 (8). The fecal samples were also examined for the presence of other enteric pathogens, including parasites and helminths. Plasma samples were collected from all patients on days 3 (acute-phase sample) and 9 (convalescent-phase sample) after hospitalization.

Two groups of vaccinees were studied: Bangladeshi and Swedish adults, who, after giving informed consent to participate in the study, each received two doses of an oral ETEC vaccine in bicarbonate buffer 2 weeks apart (17). Each vaccine dose consisted of formalin-inactivated ETEC bacteria expressing the colonization factors CFA/I and CS1 to CS6 (i.e., 2×10^{10} each of CFA/I^+, $CS1^+$, $CS2^+$ $CS3^+$, $CS4^+$ $CS6^+$, and $CS5^+$ $CS6^+$ bacteria, respectively) and 1 mg of recombinant cholera toxin B subunit (SBL Vaccin AB, Stockholm, Sweden). Plasma and fecal samples were collected on days 0 and 21—i.e., prior to vaccination and 7 days after the second vaccine dose—from the Bangladeshi volunteers, while serum and fecal samples were obtained from the Swedish volunteers on days 0 and 23 (9 days after the second vaccination).

An immunoblot assay in which CS6 purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used as the antigen was established for detection of CS6-specific immunoglobulin A (IgA) antibodies in the clinical specimens (8). In some immunoblotting experiments, heat extracts of the CS6-expressing strain E11881/14 ($CS4^-$ $CS6^+$ ST^- LT^- O25:H42) (14) or the $CS6^-$ isogenic mutant E11881/2 ($CS4^-$ $CS6^-$ ST^+ LT^+ O25:H42) (14) were used as the antigen. Bacterial heat extracts were prepared by heating bacterial suspensions at 60°C for 30 min and harvesting the supernatant following centrifugation (6). CS6 was purified from heat extracts of the CS6-only strain E11881/14 by electroelution of a single band, corresponding to the molecular weight of CS6, from an SDS-polyacrylamide gel (6). For control purposes, CS5-specific antibodies were similarly analyzed, using an immunoblot assay in which CS5, purified from strain E17018A ($CS5^+$ $CS6^+$ ST^+ LT^- O167:H5) (14) as previously described (3), was used as the antigen. Plasma and serum samples were tested at a final dilution of 1:60 as well as threefold dilutions thereof. Fecal samples were extracted as previously described (10), and the acute- and convalescent-phase fecal extracts from patients were tested at a dilution of 1:2. Fecal

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Göteborg University, Guldhedsgatan 10A, 413 46 Göteborg, Sweden. Phone: 46 31 60 47 19. Fax: 46 31 82 69 76. E-mail: anna.helander@microbio.gu.se.

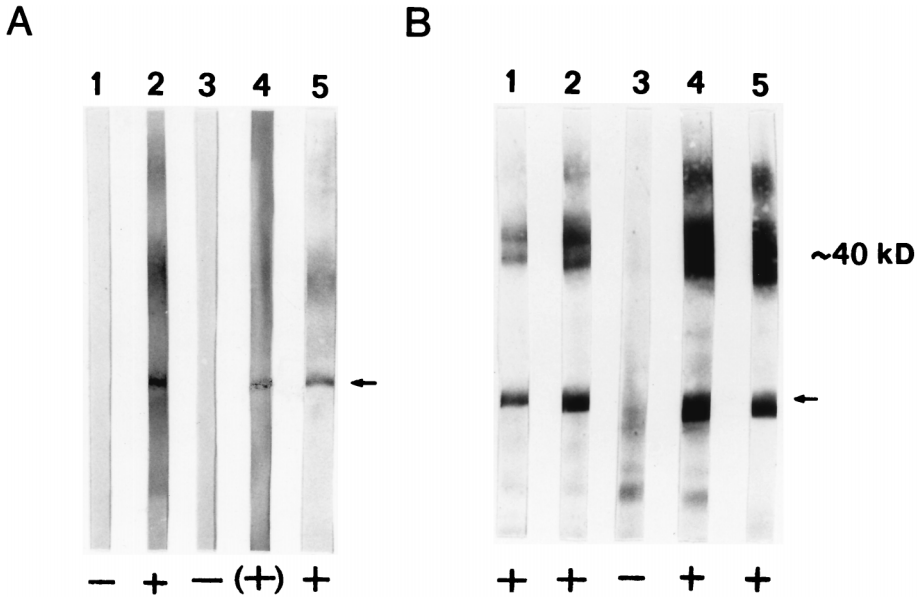

FIG. 1. Immunoblot showing CS6-reactive IgA antibodies in fecal extracts (A) and plasma (B) of Bangladeshi adults with natural $CS5^+$ $CS6^+$ ETEC infections. (A) Feces was obtained from patient ETP-009 on the day of admission to the hospital (day 0) (lane 1) and 9 days later (lane 2) and from patient ETP-010 on days 0 (lane 3) and 9 (lane 4). (B) Plasma samples were collected from patients ETP-009 and ETP-010 3 (lanes 1 and 3, respectively) and 9 (lanes 2 and 4, respectively) days after hospitalization. The arrow indicates the position of the CS6 band as determined by using a CS6-specific MAb (CS6-20:11:9) (lane 5). The symbols at the bottom of the figure correspond to the intensity grading of the CS6 band [−, no reactivity; (+), weak reactivity; and +, strong reactivity].

extracts of the day 0 specimens from the vaccinees were diluted 1:2, and corresponding postvaccination samples were tested at dilutions adjusted to the same total IgA concentrations as the preimmune samples. Only antibodies of the IgA isotype were determined, since previous studies have shown that vaccination and natural infection predominantly give rise to anti-CF responses of this isotype in the intestine and that IgA anti-CF responses in serum are more frequent and of larger magnitude than corresponding IgG responses (1, 16–18). Total IgA levels in the fecal extracts were measured by ELISA (19).

In initial experiments, we attempted to determine CS6-specific IgA antibodies in the different specimens by ELISA tech-

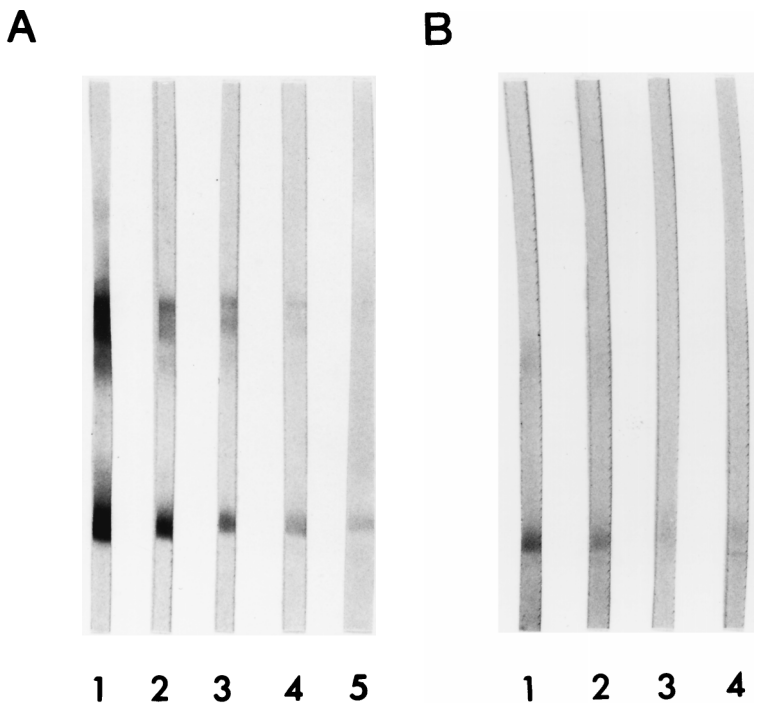

FIG. 2. Immunoblot showing CS6-reactive IgA antibodies in plasma or serum collected from a Bangladeshi adult with a natural $CS5^+$ $CS6^+$ ETEC infection 9 days after admission to the hospital (ETP-009) (A) and from a Swedish vaccinee postvaccination (Ec-215) (B). The specimens were diluted 1:60 (lane 1), 1:180 (lane 2), 1:540 (lane 3), 1:1,620 (lane 4), and 1:4,860 (lane 5).

TABLE 1. Local and systemic IgA responses to CS6 and CS5 among patients and vaccinees as determined by immunoblotting

Patient or vaccinee	CF profile of infecting strain	IgA response evident in:							
		Fecal extract				Plasma/serum			
		CS6[a]		Increase[b]		CS6[a,c]		Increase[b]	
		Acute phase (pre)	Convalescent phase (post)	CS6	CS5	Acute phase (pre)	Convalescent phase (post)	CS6	CS5
Bangladeshi patients									
ETP-007	$CS5^+$ $CS6^+$	−	+	+		(+) [ND]	+ [ND]	+	+
ETP-009	$CS5^+$ $CS6^+$	−	+	+	+	+ [ND]	+ [>1:4,860]		
ETP-010	$CS5^+$ $CS6^+$	−	(+)	+	+	−	+ [>1:4,860]	+	+
ETP-015	$CS5^+$ $CS6^+$	ND	ND			−	+ [>1:4,860]	+	+
ETP-018	$CS5^+$ $CS6^+$	−	−		+	−	−		+
ETP-021	$CS5^+$ $CS6^+$	−	+	+		(+) [1:60]	+ [1:180]	+	+
ETP-006	CFA/I^+	+	+			−	−		
ETP-011	CFA/I^{+d}	−	+	+	+	(+) [1:60]	(+) [1:60]		
ETP-020	CFA/I^+	−	+	+		(+) [1:60]	(+) [1:60]		+
ETP-014	$CS1^+$ $CS3^+$	+	+			−	−		
Vaccinees[e]									
Bangladeshi									
ETV-001	NA[f]	(+)	+	+		(+) [1:180]	(+) [1:180]		
ETV-006	NA	−	(+)	+	+	−	+ [1:180]	+	
ETV-012	NA	−	−			(+) [1:180]	+ [1:180]	+	+
ETV-018	NA	−	(+)	+		−	+ [1:180]	+	
ETV-024	NA	(+)	+	+		−	+ [1:60]	+	+
Swedish									
EC-205	NA	−	−			−	+ [1:180]	+	+
EC-210	NA	−	(+)	+	+	−	−		
EC-215	NA	−	(+)	+		(+) [1:60]	+ [1:540]	+	+
EC-221	NA	−	−		+	−	(+) [1:60]	+	
EC-240	NA	−	(+)	+		(+) [1:60]	(+) [1:60]		+

[a] Acute-phase specimens were collected on day 0 (fecal specimens) or day 3 (plasma specimens), and convalescent-phase specimens were collected on day 9 after hospitalization. Prevaccination specimens (pre) were collected prior to vaccination, and postvaccination specimens (post) were collected 7 to 9 days after the second vaccination. −, no reactivity; (+), weak reactivity; +, strong reactivity against purified CS6; ND, not determined.

[b] +, increase evident.

[c] Values in brackets are the highest dilutions of the plasma or serum samples that gave positive reactions in the immunoblot assay.

[d] Coinfected with *Giardia lamblia* cysts.

[e] Vaccinated with ETEC vaccine (lot E0001).

[f] NA, not applicable.

niques, using the CS6 preparation purified by SDS-PAGE or whole $CS6^+$ and corresponding $CS6^-$ bacteria as solid-phase antigens. We also tried to establish a sandwich ELISA in which plates were coated with CS6-specific MAbs followed by SDS-PAGE-purified CS6 (15). In none of these instances could specific antibody titers against CS6 or a difference in specific IgA content between acute- and convalescent-phase or pre- and postvaccination samples from the different individuals be detected. Due to problems in developing a suitable ELISA with the CS6 antigens available, we established an immunoblot assay, employing our SDS-PAGE-purified CS6 preparation as the antigen, for the qualitative assessment of specific antibodies in the clinical specimens. Since the various sera and fecal samples gave rise to CS6 bands of various intensities in the immunoblot (Fig. 1), we graded the intensity of the CS6 bands as weak or strong. A shift from no response in the acute-phase/prevaccination sample to a weak or strong response in the convalescent-phase/postvaccination sample or from a weak acute-phase/prevaccination response to a strong convalescent-phase/postvaccination response was considered to be a significant CS6-specific antibody response. We also attempted to do a semiquantitative analysis of the anti-CS6 antibody responses detected in the immunoblot assay by testing different dilutions of the plasma and serum samples (Fig. 2).

Fecal CS6-specific IgA antibodies were detected in the day 9 samples by the immunoblot assay, using SDS-PAGE-purified CS6 as the antigen, in four of five patients infected with $CS5^+$ $CS6^+$ ETEC (Table 1; Fig. 1A). Five of the six $CS5^+$ $CS6^+$ ETEC-infected patients developed bands specific for CS6 on analysis of the convalescent-phase plasma samples; in four of these patients, there was an increase in specific IgA on day 9 compared to the level found on day 3 (Table 1; Fig. 1B; Fig. 2A). All of the $CS5^+$ $CS6^+$ ETEC-infected patients had CS5-reactive antibodies in their convalescent-phase plasma and fecal extract specimens, including the patient not responding to CS6 (ETP-018), as determined by immunoblotting with purified CS5 as the antigen.

Surprisingly, the CS6-specific IgA reactivities of the convalescent-phase fecal specimens from two of the three individuals infected with CFA/I-expressing bacteria were higher than those of their acute-phase samples; none of these individuals had a higher level of CS6 reactivity in the convalescent-phase plasma specimen than in the acute-phase sample (Table 1). The convalescent-phase fecal specimens did not recognize antigens with a molecular weight corresponding to that of CS6 when heat extract from the $CS6^-$ strain E11881/2 was used as the antigen in immunoblotting, indicating the specificity of the reaction. Furthermore, the increased CS6 responses did not seem to be due to mixed ETEC infections, since only CFA/I-expressing bacterial strains were isolated from the feces of the patients. The $CS1^+$ $CS3^+$ ETEC-infected patient had similar levels of CS6-reactive antibodies in feces before and after infection, whereas no specific IgA was found in the plasma (Table 1). However, there was an increase in CS1-specific IgA in the late plasma specimen compared to that in the sample collected earlier (data not shown).

Four of the five Bangladeshi vaccinees showed an increase in CS6-specific IgA in their feces after vaccination with an oral

ETEC vaccine containing formalin-inactivated CS6$^+$ bacteria, and the plasma specimens of four of them also showed a response (Table 1). In addition, three of the five Swedish vaccinees had increased fecal anti-CS6 IgA levels on day 23 compared to their day 0 levels, and three vaccinees had higher antibody levels in their sera on day 23 than on day 0 (Table 1; Fig. 2B).

In conclusion, we have shown that CS6 is immunogenic in humans after natural infection, giving rise to local—i.e., intestinal—as well as systemic IgA responses reflected by increased antibody levels at a late stage of infection, compared to those found at an early stage. Furthermore, the whole-cell component of an oral inactivated ETEC vaccine containing formalin-inactivated CS6$^+$ bacteria seemed to induce both local and systemic immunity in both Bangladeshi and Swedish volunteers. Comparable titers in plasma and serum were recorded postvaccination in both the Bangladeshi and Swedish vaccinees. However, these titers were generally of lower magnitude than those observed postinfection in the CS6-infected Bangladeshi patients (Table 1).

The reason that most of the Bangladeshi adults had CS6-reactive antibodies in both serum and feces early after infection or even prior to immunization, as reported previously for other CFs (1, 16), is probably that volunteers living in an area in which ETEC is endemic are continuously exposed to subclinical ETEC infections, some of which may be caused by CS6$^+$ strains. Alternatively, since some of the acute-phase samples from the patients may have been collected 3 to 5 days after the initiation of infection with the ETEC strain, and considering that the incubation time of ETEC infection ranges from 1 to 5 days (median, 2 days) (9), there is ample time for a primed immune system to initiate an immune response (18). Surprisingly, antibodies reactive with CS6 were observed also in some of the Swedish preimmune sera. These antibodies may have been directed at impurities of the same molecular weight as CS6 in the immunoblot assay. However, since the sera did not recognize any antigens with a molecular weight corresponding to that of CS6 when a heat extract of the CS6$^-$ strain E11881/2 (i.e., the CS6$^-$ mutant corresponding to the strain from which CS6 was purified) was used as the antigen in the immunoblot assay, this explanation is perhaps less likely. Instead, the reactivity demonstrated in the Swedes may be due to the presence of antibodies cross-reacting with CS6. As evident from Fig. 1, bands corresponding to a molecular mass of approximately 40 kDa developed when some of the specimens were tested in the CS6 immunoblot assay. However, these bands were also identified by the CS6-specific MAb, suggesting that they represent either multimers of CS6 or CS6 subunits associated with another bacterial constituent. Neither 2-mercaptoethanol, dithiothreitol, nor 6 M urea treatment of the purified CS6 prior to its application to the SDS-PAGE gel had any effect on the binding of the CS6-specific MAb to the 40-kDa band, suggesting that it is not a multimer of CS6 subunits.

In summary, we have shown that CS6 is immunogenic in humans both after natural infection and after vaccination with an oral inactivated ETEC vaccine containing formalin-inactivated CS6$^+$ bacteria, giving rise to local as well as systemic IgA responses.

Financial support for this work was obtained from the Swedish Medical Research Council (grant 16X-09084), the Swedish Agency for Research Cooperation with Developing Countries, and the World Health Organization.

Editor: J. R. McGhee

REFERENCES

1. **Åhrén, C., C. Wennerås, J. Holmgren, and A.-M. Svennerholm.** 1993. Intestinal antibody response after oral immunization with a prototype cholera B subunit-colonization factor antigen enterotoxigenic *Escherichia coli* vaccine. Vaccine **11:**929–934.
2. **Binsztein, N., M. J. Jouve, G. I. Viboud, L. López Moral, M. Rivas, I. Ørskov, C. Åhrén, and A.-M. Svennerholm.** 1991. Colonization factors of enterotoxigenic *Escherichia coli* isolated from children with diarrhea in Argentina. J. Clin. Microbiol. **29:**1893–1898.
3. **Evans, D. G., D. J. Evans, Jr., S. Clegg, and J. A. Pauley.** 1979. Purification and characterization of the CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect. Immun. **25:**738–748.
4. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18:**330–337.
5. **Gaastra, W., and A.-M. Svennerholm.** 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). Trends Microbiol. **4:**444–452.
6. **Grewal, H. M. S., A. Helander, A.-M. Svennerholm, M. K. Bhan, W. Gaastra, and H. Sommerfelt.** 1994. Genotypic and phenotypic identification of coli surface antigen 6-positive enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **32:**1295–1301.
7. **Helander, A., G. C. Hansson, and A.-M. Svennerholm.** 1997. Binding of enterotoxigenic *Escherichia coli* to isolated enterocytes and intestinal mucus. Microb. Pathog. **23:**335–346.
8. **Helander, A., H. M. S. Grewal, W. Gaastra, and A.-M. Svennerholm.** 1997. Detection and characterization of the coli surface antigen 6 of enterotoxigenic *Escherichia coli* by using monoclonal antibodies. J. Clin. Microbiol. **35:**867–872.
9. **Levine, M. M., D. R. Nalin, D. L. Hoover, E. J. Bergquist, R. B. Hornick, and C. R. Young.** 1979. Immunity to enterotoxigenic *Escherichia coli*. Infect. Immun. **23:**729–736.
10. **Li, A., T. Pál, U. Forsum, and A. A. Lindberg.** 1992. Safety and immunogenicity of the live oral auxotrophic *Shigella flexneri* SFL124 in volunteers. Vaccine **10:**395–404.
11. **Lopez-Vidal, Y., P. Klemm, and A.-M. Svennerholm.** 1988. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin-producing *Escherichia coli*. J. Clin. Microbiol. **26:**1967–1972.
12. **Lopez-Vidal, Y., and A.-M. Svennerholm.** 1990. Monoclonal antibodies against the different subcomponents of colony factor antigen II of enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **28:**1906–1912.
13. **McConnell, M. M., H. Chart, A. M. Field, M. Hibberd, and B. Rowe.** 1989. Characterization of a putative colonization factor (PCFO166) of enterotoxigenic *Escherichia coli* of serogroup O166. J. Gen. Microbiol. **135:**1135–1146.
14. **McConnell, M. M., L. V. Thomas, G. A. Willshaw, H. R. Smith, and B. Rowe.** 1988. Genetic control and properties of coli surface antigens of colonization factor antigen IV (PCF8775) of enterotoxigenic *Escherichia coli*. Infect. Immun. **56:**1974–1980.
15. **Qadri, F., G. Jonson, Y. A. Begum, C. Wennerås, M. J. Albert, M. A. Salam, and A.-M. Svennerholm.** 1997. Immune response to the mannose-sensitive hemagglutinin in patients with cholera due to *Vibrio cholerae* O1 and O139. Clin. Diagn. Lab. Immunol. **4:**429–434.
16. **Stoll, B. J., A.-M. Svennerholm, L. Gothefors, D. K. Barua, S. Huda, and J. Holmgren.** 1986. Local and systemic antibody responses to naturally acquired enterotoxigenic *Escherichia coli* diarrhea in an endemic area. J. Infect. Dis. **153:**527–534.
17. **Svennerholm, A.-M., C. Åhrén, and M. Jertborn.** 1997. Vaccines against enterotoxigenic *Escherichia coli* infections. I. Oral inactivated vaccines against enterotoxigenic *Escherichia coli*, p. 865–873. *In* M. M. Levine, G. C. Woodrow, J. B. Kaper, and G. S. Cobon (ed.), New generation vaccines, 2nd ed. Marcel Dekker, Inc., New York, N.Y.
18. **Svennerholm, A.-M., and J. Holmgren.** 1995. Oral B-subunit whole-cell vaccines against cholera and enterotoxigenic *Escherichia coli* diarrhea, p. 205–232. *In* D. A. A. Ala'aldeen and C. E. Hormaeche (ed.), Molecular and clinical aspects of bacterial vaccine development. John Wiley & Sons, New York, N.Y.
19. **Svennerholm, A.-M., M. Jertborn, L. Gothefors, A. M. M. M. Karim, D. A. Sack, and J. Holmgren.** 1984. Mucosal anti-toxic and anti-bacterial immunity after cholerae disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149:**884–893.
20. **Svennerholm, A.-M., Y. Lopez Vidal, J. Holmgren, M. M. McConnell, and B. Rowe.** 1988. Role of PCF8775 antigen and its coli surface subcomponents for colonization, disease, and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect. Immun. **56:**523–528.

20a. **Svennerholm, A.-M.** Unpublished data.

21. **Wolf, M. K., D. N. Taylor, E. C. Boedeker, K. C. Hyams, D. R. Maneval, M. M. Levine, K. Tamura, R. A. Wilson, and P. Echeverria.** 1993. Characterization of enterotoxigenic *Escherichia coli* isolated from U.S. troops deployed to the Middle East. J. Clin. Microbiol. **31:**851–856.