

Immune Response to the Mannose-Sensitive Hemagglutinin in Patients with Cholera Due to *Vibrio cholerae* O1 and O139

FIRDAUSI QADRI,^{1*} GUNHILD JONSON,^{2†} YASMEEN ARA BEGUM,¹ CHRISTINE WENNERÅS,²
M. JOHN ALBERT,¹ M. ABDUS SALAM,¹ AND ANN-MARI SVENNERHOLM²

*International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka 1000, Bangladesh,¹ and
Department of Medical Microbiology and Immunology, University of Göteborg, Guldhedsgatan 10,
S-413 46 Göteborg, Sweden²*

Received 27 February 1997/Accepted 20 March 1997

The mannose-sensitive hemagglutinin (MSHA) is a type 4 pilus present in *Vibrio cholerae* O1 strains of the El Tor biotype, as well as in strains of serogroup O139. It has been shown to be a colonization antigen in animal models. The aim of this study was to investigate systemic and local antibody responses to MSHA in adult patients with cholera due to *V. cholerae* O1 and O139. Twenty-four of 28 (86%) patients with O1 cholera and 11 of 17 (65%) patients with O139 cholera showed significant increases in MSHA-specific immunoglobulin A (IgA) and IgM antibody-secreting cells (ASCs) 7 days after the onset of disease. However, the magnitude of the ASC response in O1 cholera patients was significantly higher than that in the O139 cholera patients in both IgA-producing ($P = 0.015$) and IgM-producing ($P = 0.029$) cells. Both groups of patients responded with antibody responses to MSHA in plasma, seroconverting with both IgA (63 to 70% of patients) and IgG (43 to 59% of patients) antibodies. Compared to the MSHA-specific antibody levels determined in healthy controls ($n = 10$), more than 90% of O1 and O139 cholera patients showed responses to MSHA of both the IgA and the IgG isotypes. About 70% of the patients in both groups also had antibody responses to MSHA in their feces. In summary, we demonstrated that MSHA is immunogenic, giving rise to both systemic and local antibodies in patients with cholera due to both O1 and O139 serogroups.

Recently, interest has been focused on a number of colonization factors and putative adhesins in *Vibrio cholerae* O1 organisms (9, 11, 31). Among these factors, the toxin-coregulated pilus (TCP) first detected in *V. cholerae* O1 of the classical biotype (31) and subsequently in El Tor vibrios (33) has been shown to be important for bacterial colonization of the intestine (5, 32). TCP has also been found to be a colonization factor for the newly identified epidemic serogroup of *V. cholerae* O139 (3, 4, 31). A second pilus antigen is the mannose-sensitive hemagglutinin (MSHA), which is associated with *V. cholerae* serogroup O1 strains of the El Tor biotype (11, 14). It is a type 4 pilus and consists of 17.4-kDa protein subunits (12). Studies with animal models have suggested that MSHA is immunogenic (19, 20). However, its role in colonization in infant mice has been controversial. Thus, some studies (5, 32) have indicated that TCP but not MSHA is critical for colonization in this model, whereas Osek et al. (19) and Mukhopadhyay et al. (17) have shown that MSHA also has a role in colonization. *V. cholerae* strains of the O139 serogroup also produce MSHA (13, 18, 26). The nucleotide sequence of the *mshA* gene in a *V. cholerae* O139 strain tested was identical to the sequence previously described for an O1 El Tor strain (12), and there is an indication of cross-reactivity between MSHA in O1 and O139 vibrios (13).

This study was initiated to define the role of MSHA in natural disease in a region where cholera is endemic, specifically regarding its immunogenic potentials. For this purpose, the immune responses of adult patients with cholera caused by *V. cholerae* O1 or *V. cholerae* O139 were studied. MSHA-

specific antibody-secreting cells (ASCs) in peripheral blood were determined and compared with the antibody responses in plasma and fecal extracts.

MATERIALS AND METHODS

Study group. Twenty-seven patients with cholera caused by *V. cholerae* O139 and 30 patients with cholera caused by *V. cholerae* O1 El Tor (27 with *V. cholerae* O1 Ogawa infections and 3 with *V. cholerae* O1 Inaba infections) were recruited from November 1993 to January 1996. The patients with *V. cholerae* O139 infection were 21 to 45 years of age (median, 30 years) and had either severe (76% cases) or moderate (24% cases) dehydration (36) with a history of 4 to 16 h (median, 8 h) of diarrhea prior to hospitalization. The patients with cholera caused by *V. cholerae* O1 were 18 to 42 years of age (median, 25 years) and had severe (82% of patients) or moderate (18% of patients) dehydration and a history of 4 to 14 h (median, 9 h) of diarrhea before hospitalization. The *V. cholerae* O1 and O139 patients were matched in all respects. Ten adult males in the same age group as the patients (age range, 18 to 40 years; median age, 24.5 years) with no history of diarrhea during the previous 6 months were also included in the study.

Confirmation of bacterial strains. The stools of patients suffering from acute watery diarrhea were examined by dark-field microscopy for the darting movement of vibrios (6) and inhibition of this movement by antibodies specific for the *V. cholerae* O1 (24) or O139 (21) serogroup. Stools were also plated onto taurocholate-tellurite-gelatin agar (16) and gelatin agar (Difco, Detroit, Mich.) for the detection of vibrios. After overnight incubation, serological confirmation of vibrio-like colonies was carried out by slide agglutination with rabbit polyclonal sera specific for *V. cholerae* O1 (as well as the Inaba and Ogawa serotypes) and with a monoclonal antibody (MAb), MAb ICL11, directed against *V. cholerae* O139 (21). Stools were also cultured to detect other enteric pathogens, such as *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. (35). Stools were also tested by direct microscopy for the detection of cyst and vegetative forms of parasites as well as ova of helminths.

Campylobacter jejuni was isolated from one O1 cholera patient. Stool microscopy revealed the presence of a few ova of *Ascaris lumbricoides* in one O1 cholera patient and three O139 cholera patients. No other enteric pathogens were detected in the stool specimens.

Sample collection. After microbiological confirmation of cholera infection, venous blood and feces were collected from patients at the acute stage of disease, i.e., the second day of hospitalization, which was considered to be approximately 2 days after the onset of diarrhea (day 2). Blood was also collected 5, 9, and 20 days later, during convalescence (that is, at 7, 11, and 22 days after the onset of

* Corresponding author. Mailing address: Laboratory Sciences Division, ICDDR,B, GPO Box 128, Dhaka 1000, Bangladesh. Phone: 880 2 871751 to 871760. Fax: 880 2 872529, 880 2 883116, or 880 2 886050. E-mail: fqadri@cholera.bangla.net.

† Deceased. We dedicate this report to her memory.

illness, respectively) (22). Single blood samples were collected from healthy controls.

MNCs, plasma, and ELISPOT assays. Peripheral blood mononuclear cells (MNCs) were isolated from heparinized venous blood on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Plasma collected from the top of the Ficoll gradient was stored in aliquots at -20°C . The specific ASC responses of the immunoglobulin A (IgA), IgM, and IgG isotypes were determined by the two-color ELISPOT procedure (7, 34). ASC responses to MSHA were determined by the sandwich ELISPOT technique with a well-characterized MSHA-specific mouse MAb, MAb 17:10 (30). The MAb is specific for MSHA and does not recognize lipopolysaccharide (LPS). As a result, anti-LPS antibodies do not react in this procedure, in which plates are coated with the antibody (11). Individual wells of 96-well plates (Millititer HA; Millipore Corp., Bedford, Mass.) with nitrocellulose on the bottom were coated with MAb 17:10 (at a concentration of $4\ \mu\text{g}/\text{ml}$ [$100\ \mu\text{l}/\text{well}$] overnight at 4°C). After washing the MAb-coated plates twice with phosphate-buffered saline (PBS; $10\ \text{mM}$; pH 7.2), a purified MSHA preparation (protein content, $3.6\ \mu\text{g}/\text{ml}$; $100\ \mu\text{l}/\text{well}$) was used as capture antigen (11), and the plates were incubated at 37°C for 1 h. After washing three times with PBS, MNC suspensions were applied to wells at different concentrations (5×10^4 to 1×10^6) and the plates incubated for 4 h at 37°C in a humidified atmosphere supplemented with 5% CO_2 . Then, a mixture of two affinity-purified goat anti-human immunoglobulin antibodies, one conjugated to alkaline phosphatase and the other conjugated to horseradish peroxidase (Southern Biotechnology Association, Birmingham, Ala.), was added to the wells by following the procedures described earlier (34). Total IgA-, IgG-, and IgM-secreting cells were detected by using wells coated with affinity-purified goat antibodies directed against the F(ab')₂ fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.), which recognizes all three isotypes. Spots were counted in quadruplicate wells, and data were expressed as the number of spot-forming cells/ 10^6 MNCs. The numbers of ASCs were calculated for samples collected from patients only on day 2, day 7, and day 11 after the onset of disease. A fourfold or higher increase in the number of MSHA-specific ASCs/ 10^6 MNCs from that seen at the acute stage was considered a significant response. Twenty-eight patients with O1 cholera O1, 17 patients with O139 cholera, and 10 healthy adult controls were studied for the MSHA-specific ASC response.

Preparation of fecal extracts. Stool specimens were collected from patients at the acute stage (day 2) and the convalescence stage (days 7, 11, and 22) and were frozen immediately at -70°C . Fecal extracts were prepared by mixing 4 g of thawed stool with 16 ml of a PBS-supplemented solution with Tween 20 (0.05%), soybean trypsin inhibitor (100 mg/ml), EDTA (0.05 M), and phenylmethylsulfonyl fluoride (10 mM). The suspension was allowed to stand for 15 min with intermittent shaking, followed by filtration through cheesecloth to remove particulate materials. It was then centrifuged at $20,000 \times g$ for 30 min. The supernatant was frozen in aliquots at -70°C after the addition of bovine serum albumin (final concentration, 0.1%) and sodium azide (final concentration, 0.02%). Total IgA contents were determined by enzyme-linked immunosorbent assay (ELISA) with pooled human Swedish milk with a known IgA concentration of 1 mg/ml as a standard (28). The MSHA-specific response was expressed as the number of units per microgram and was calculated by dividing the titers (in units per milliliter) by the total IgA concentration (in micrograms per milliliter) and multiplying by 10 (2). Specimens with total IgA contents of $<20\ \mu\text{g}/\text{ml}$ were excluded from the analyses. Acute- and convalescent-phase samples in which the total IgA concentration varied more than 10-fold were also excluded (1). On the basis of these exclusion criteria, only 21 fecal samples from O1 cholera patients and 21 fecal samples from O139 cholera patients could be used to analyze MSHA-specific fecal antibody responses. A twofold or greater increase in the MSHA-specific titers (units per microgram) between acute- and convalescent-stage samples was considered a significant response.

MSHA sandwich ELISA. MAb 17:10 (concentration, $2\ \mu\text{g}/\text{ml}$; $100\ \mu\text{l}/\text{well}$ in PBS) was applied to microtiter plates (Nunc, Roskilde, Denmark), and the plates were incubated overnight at 4°C . After washing the plates with PBS and blocking with 1% bovine serum albumin in PBS, incubation was carried out for 30 min at 37°C . After washing with PBS, MSHA was applied as capture antigen (protein concentration, $1.8\ \mu\text{g}/\text{ml}$; $100\ \mu\text{l}/\text{well}$). Plasma and fecal antibodies diluted in 0.1% bovine serum albumin-PBS containing 0.05% Tween were serially diluted threefold in microtiter plates, and the plates were incubated for 90 min at room temperature. Affinity-purified rabbit anti-human immunoglobulin antibodies with IgA or IgG specificity conjugated to horseradish peroxidase were used as secondary antibodies (Jackson ImmunoResearch Laboratories Inc.), and the plates were developed with *ortho*-phenylenediamine (10 mg), prepared by dissolving the *ortho*-phenylenediamine in 10 ml of 0.1 M sodium citrate buffer (pH 4.5) to which $4\ \mu\text{l}$ of 30% hydrogen peroxide ($4\ \mu\text{l}/10\ \text{ml}$) was added just before use. The optical density was measured after 20 min at 490 nm after the addition of $25\ \mu\text{l}$ of 1 M sulfuric acid per well. The ELISA was found to be specific for MSHA and did not recognize mouse MAbs to LPS of *V. cholerae* O1 (11) or *V. cholerae* O139 (21) tested at different dilutions (1:100 to 1:1,500). Endpoint titers were determined as the reciprocal of the interpolated dilution of the test samples giving an absorbance of 0.4 above the background absorbance. Pooled convalescent-phase plasma (day 11) obtained from four O1 cholera patients was applied in duplicate in each assay as a reference (average titers, approximately 45 for the IgA isotype and 130 for the IgG isotype). Plasma samples from the 10 healthy adults were also tested for IgA and IgG responses to MSHA to determine the

baseline levels of these antibodies. These values (geometric mean [GM] + 2 standard errors of the mean [SEMs]) were determined to be titers of 8.4 for the IgA isotype and 33 for the IgG isotype. Titers higher than these values were considered a positive response in patients. Titer calculations were carried out by using a computer-based program (Multi; DataTree Inc., Waltham, Mass.).

Detection of MSHA activity. All *V. cholerae* O1 and O139 strains isolated from patients were tested for MSHA. Strains stored at -70°C in T1N1 broth (1% Trypticase, 1% sodium chloride [pH 7.4]) containing 20% (vol/vol) glycerol were subcultured on blood agar plates and were then grown in Trypticase soy broth without glucose (Difco) for 4 to 5 h with shaking (150 rpm). Hemagglutination was performed by mixing a 2% (vol/vol) suspension of chicken erythrocytes with bacteria onto glass microscope slides as described previously (14). Inhibition of hemagglutination was assayed by using 0.5% (wt/vol) D-mannose. The presence of MSHA on the bacterial surface was confirmed by agglutination of bacteria with MAb 17:10 on microscope slides as described previously (11). *V. cholerae* El Tor Inaba strain 19479 was used as a positive MSHA control (11).

Demonstration of MshA pilin subunit and MSHA pili on *V. cholerae* O139 strains. The production of the pilin subunit by three MSHA-negative variants was determined by sodium dodecyl sulfate (SDS)-immunoblotting of whole-cell extracts by using MAb 17:10 according to the procedures described previously (12). PCR amplification of DNA was carried out with primers flanking the *mshA* gene by procedures described earlier (12).

Analyses. The Wilcoxon signed rank test, the Mann-Whitney U test, the chi-square test, and the Fisher exact test were used where applicable to evaluate the statistical significance of differences between groups. Analyses were carried out by using the statistical software SigmaStat (Jandel Scientific, San Rafael, Calif.). Comparison of the number of cholera patients with significant IgA ASC responses with plasma and fecal antibody responses was made by four-field table analyses, and the sensitivity and the predictive accuracy for antibody determinations (10) in the different assays were calculated by using the MSHA-specific IgA ASC as a reference. A *P* value of ≤ 0.05 was used as the criterion for a significant difference. The GM and SEMs of the values were calculated for all samples.

RESULTS

MSHA-specific ASCs. The ASC response to MSHA in O1 cholera patients peaked on day 7 and decreased to the acute-stage levels by day 11 after the onset of disease (Fig. 1A; Table 1). A 44-fold increase was seen for the IgA isotype (GM = 55 ASCs/ 10^6 MNCs), an 11-fold increase was seen for the IgM isotype (GM = 21 ASCs/ 10^6 MNCs), and a 5-fold increase was seen for the IgG isotype (GM = 5.2 ASCs/ 10^6 MNCs). Of the 28 *V. cholerae* O1 patients in whom ASC responses were studied, 24 responded with at least a fourfold increase in the numbers of ASCs of the IgA isotype; of the 4 who did not respond, 2 had higher ASC responses at the acute stage compared to that at the convalescent stage, and 2 showed no changes. Fewer patients responded with IgM (21 of 28) or IgG (12 of 28) ASCs.

Among the 17 O139 patients studied (Fig. 1B; Table 1), 11 had at least fourfold increases in the levels of MSHA-specific ASCs of the IgA or IgM isotype. There was no statistically significant IgG ASC response, and only one patient showed a response. When comparing peak ASC responses to MSHA between O1 and O139 cholera patients, it was found that O1 cholera patients had significantly higher responses of both the IgA (*P* = 0.010) and the IgM (*P* = 0.019) isotypes. However, there was no significant difference in the proportion of O1 and O139 cholera patients responding with MSHA-specific ASCs (of the IgA or IgM isotype). In healthy controls, no MSHA-specific ASCs of any of the three isotypes were detected.

MSHA-specific antibody responses in plasma. The antibody response to MSHA in plasma was determined by comparison of the kinetics of the response from the acute stage to that seen during different periods of the convalescent stage. The number of patients with responses was also determined by comparison of responses with those obtained in healthy Bangladeshi controls. This was done since Bangladesh is a country where cholera is endemic, and individuals might be primed and a response to antigens can be expected at an early stage of the disease. When such a comparison was carried out, it was observed that more than two-thirds of the O1 (21 of 30) and O139

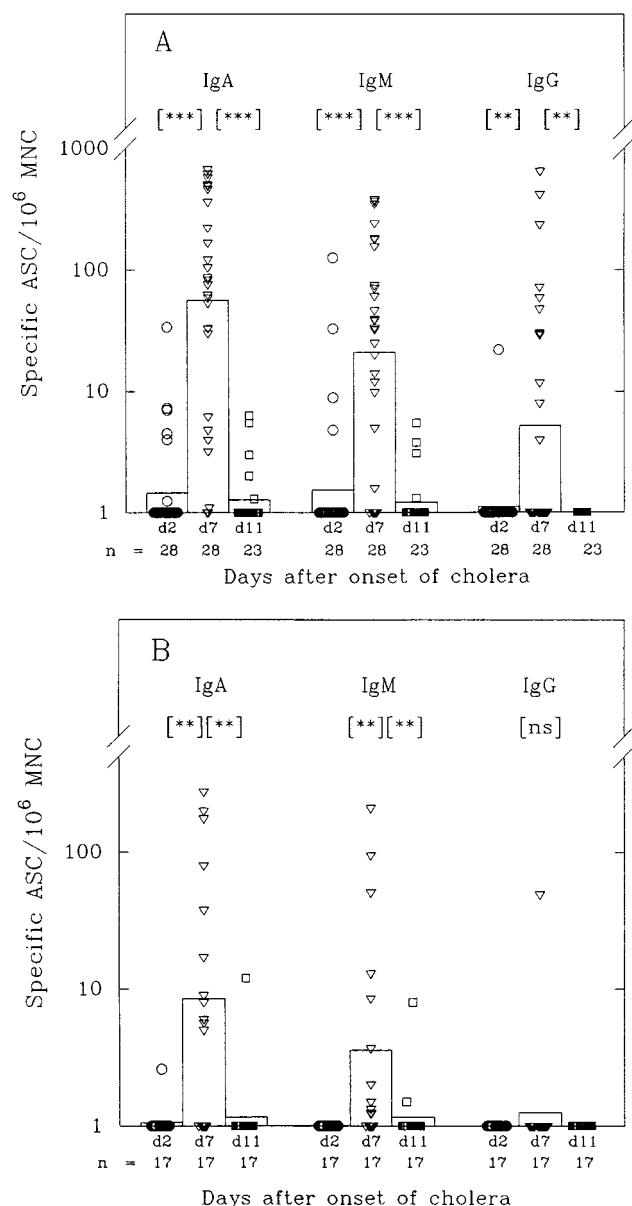


FIG. 1. The MSHA-specific ASC responses in peripheral blood of *V. cholerae* O1 (A) and *V. cholerae* O139 (B) patients at the acute stage (day 2) and later stages at day 7 and day 11 after the onset of cholera. The individual ASC responses and the GM responses (bar) are shown. Asterisks denote statistically significant differences between adjacent columns (**, <0.01 to 0.001 ; ***, <0.001); ns, not significant; n , the number of subjects studied. The Wilcoxon signed rank test or the Mann-Whitney U test was used for analyses of statistical significance.

(18 of 27) cholera patients showed a response to MSHA of the IgA isotype at the acute stage, whereas by day 11, more than 90% of patients in both groups responded. However, more O1 (87%) than O139 (37%) cholera patients showed responses of the IgG isotype at the acute stage, but by day 11 more than 94% of patients in both groups showed responses to MSHA of the IgG isotype. When the kinetics of the response was studied (Fig. 2), it was seen that significant increases in the response to MSHA of the IgA isotype was observed by day 7 in both O1 ($P = 0.009$) and O139 ($P = 0.024$) cholera patients, and these responses remained elevated until day 21. About 70% (21 of

30) of the O1 cholera patients and 63% (17 of 27) of the O139 cholera patients seroconverted during the course of the infection. The kinetics of the response of the IgG isotype in the O139 cholera patients followed a pattern similar to that of the response of the IgA isotype, and significant increases in response were observed by day 7 ($P = 0.024$), and these responses remained elevated until day 21. It was observed that 43% (13 of 30) of the O1 cholera patients and 59% (16 of 27) of the O139 cholera patients seroconverted with MSHA-specific IgG antibodies in their plasma. For the O1 cholera patients, the levels were already elevated at the acute stage, and significant increases were seen only when comparisons were made with the response at the late convalescent stage on day 21 ($P = 0.004$). However, no significant differences were seen in the plasma antibody responses to MSHA of the IgA or the IgG isotype between O1 and O139 cholera patients.

MSHA-specific IgA antibodies in fecal extracts. *V. cholerae* O1 patients (76%; 16 of 21) and *V. cholerae* O139 patients (71%; 15 of 21) responded with at least twofold increases in MSHA-specific fecal IgA antibodies. The increase in response in O1 patients was seen by day 7 (8 of 21 patients), day 11 (6 of 21), or day 22 (2 of 21) after the onset of illness. Similarly, O139 cholera patients responded with specific antibodies in feces by day 7 (9 of 21 patients), day 11 (5 of 21), or day 22 (1 of 21).

Comparison of MSHA-specific IgA ASCs with plasma and fecal antibodies of the IgA isotype. The number of patients producing significant MSHA-specific responses in plasma and fecal extracts were compared with the IgA ASC responses in blood by using four-field table analyses. When the plasma IgA antibody response was compared with the IgA ASC response in 28 O1 cholera patients, it was observed that 19 patients had both responses, one did not have an ASC response but had a plasma IgA response, 5 had positive ASC responses but negative plasma IgA responses, whereas 3 had no ASC and plasma antibody responses. Thus, the plasma IgA antibody showed a sensitivity of 79% and a predictive accuracy of 95% compared to the IgA ASC response. In 20 O1 cholera patients for whom the fecal antibody response could be compared with the IgA ASC response, 13 had both responses, whereas 2 patients had no ASC responses but had fecal antibody responses and 4 had no fecal antibody responses but MSHA-specific IgA ASC responses. This gave the fecal antibody response a sensitivity of 76% and a predictive accuracy of 87%.

For the 17 O139 cholera patients for whom MSHA-specific IgA ASC responses could be compared with the IgA response in plasma, a sensitivity of 82% and a positive predictive value of 82% were obtained. In 13 O139 cholera patients for whom a comparison of the fecal antibody response with the IgA ASC response could be made, a higher sensitivity of 90% and a positive predictive accuracy of 100% were obtained.

Comparison of MSHA expression in *V. cholerae* O139 strains with the immune response in patients. To test the hypothesis that the magnitude of the immune response to MSHA depends on the MSHA expressed on the infecting strain, all *V. cholerae* strains isolated from patients were tested for MSHA activity. Whereas 5 of 17 strains of the O139 serogroup did not agglutinate chicken erythrocytes and were not agglutinated by MSHA MAb 17:10, all the other O1 and O139 strains showed MSHA activity. Three of the five MSHA-negative O139 strains could be further tested for the presence of MSHA pili, and all were found to lack the pili as tested by immunoelectron microscopy with MAb 17:10. However, Western blot (immunoblot) analysis showed that the three strains were positive for the MshA pilin subunit. Amplification by PCR showed that the strains were also positive for the *mshA*

TABLE 1. Immune response to MSHA in O1 and O139 cholera patients

Response and antibody	<i>V. cholerae</i> O1				<i>V. cholerae</i> O139			
	GM	Range (GM \pm SEM)	Frequency of response ^a	<i>P</i> value	GM	Range (GM \pm SEM)	Frequency of response ^a	<i>P</i> value
ASCs ^b								
IgA	55	36–82	24/28	<0.001	9	5–14	11/17	0.001
IgG	5	3–8	12/28	0.01	1.3	1–1.6	1/17	NS ^c
IgM	21	13–34	21/28	<0.001	3.6	2.3–5.6	5/17	0.001
Plasma antibody ^d								
IgA	43	30–62	29/30	0.001	44	32–59	23/27	0.006
IgG	371	284–483	27/30	0.004	211	161–275	21/27	0.002
Fecal antibody ^e	4.2	3–5.7	16/21	0.002	9.0	5.5–14	15/21	0.001

^a Data indicate number responding/number tested.

^b The GM peak ASC response (number of ASCs/10⁶ MNCs) on day 7 is presented, and comparisons are made with the response seen at the acute stage.

^c NS, not significant.

^d GM plasma antibody response in plasma is shown for IgA (day 11) and IgG (day 22). A responder is defined as a patient with a titer greater than the GM + 2 SEMs observed in healthy volunteers (titers, 8.4 for the IgA isotype and 33 for the IgG isotype) during the study period.

^e MSHA-specific antibodies determined in feces. A responder is defined as a person with a twofold or greater increase in response from the acute stage to the convalescent stage at either day 7, 11, or 22 after the onset of disease.

gene. Only two of the five patients infected with these MSHA-negative O139 strains could be studied for the ASC response, and both patients were found to be nonresponders. Neither the plasma (both IgA and IgG) nor the feces of these two patients showed significant responses. However, of the other three patients, one responded with fecal antibody of the IgA isotype and two responded with plasma antibody of the IgG isotype. None of the five patients infected with the MSHA-negative variant of O139 responded with the IgA isotype in plasma.

DISCUSSION

Our study showed that both O1 and O139 cholera patients responded with local and systemic antibodies to MSHA, in addition to circulating B cells. In cholera, which is a noninvasive disease, the antitoxic and antibacterial secretory antibodies present in the intestinal lumen are believed to offer protection against disease by neutralizing the effects of cholera toxin and inhibiting colonization (28). However, measuring antibodies in intestinal secretions, especially in feces, is associated with a number of problems, including proteolytic degradation of antibodies (8). In this study, we have therefore attempted to study the mucosal immune response to MSHA using enzyme-inactivated fecal extracts as well as specific ASC responses in the circulation. The transient appearance of ASCs in blood has been found to be a useful proxy measure of the gut immune response after enteric infection (15, 25) or oral immunization with enteric vaccines (23, 34). Furthermore, since antibodies in the serum, both antitoxic and antibacterial, have been found to be good correlates of the mucosal immune response in patients, we have also studied the MSHA-specific antibody response in plasma (28). A majority of both O1 and O139 cholera patients responded, producing significant amounts of MSHA-specific circulating ASCs of the IgA isotype, IgA titers in plasma, and fecal IgA antibodies. In both O1 and O139 cholera patients, MSHA-specific fecal antibodies showed a sensitivity of 76 to 90% in comparison to the IgA ASC response, as well as a high predictive accuracy of 87 to 100% for determining an ASC response. The fecal antibodies can therefore be used to assess the local antibody response to MSHA in the gut. The plasma IgA antibodies appeared to be somewhat more accurate in O1 cholera patients than in O139 cholera patients for predicting an ASC response. However,

since the sensitivity was about 80%, plasma will still be a good extraintestinal fluid marker for indirectly measuring the local antibody response. The response of ASCs in the circulation is a proxy measure of the local immune response in the gut. This may be replaced by testing plasma or feces for MSHA antibodies in field studies; however, whether this will stand true for vaccine studies is unclear (10).

Interestingly, whereas significant ASC responses of all three isotypes were seen in O1 cholera patients, O139 cholera patients had only IgA and IgM isotype responses. The magnitude

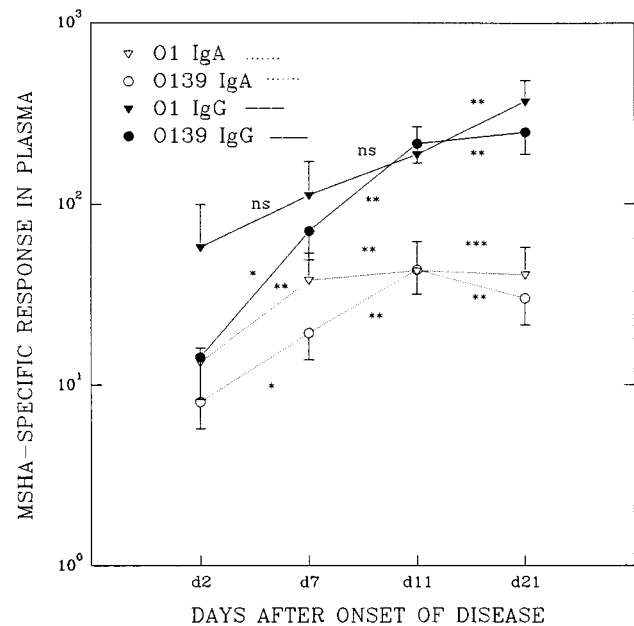


FIG. 2. The MSHA-specific IgA and IgG antibody responses in plasma of *V. cholerae* O1 and O139 patients at the acute stage (day 2) and convalescent stages (days 7, 11, and 22) after the onset of cholera. Values are GMs, and lines indicate \pm 1 SEM. Asterisks denote statistically significant differences between the acute stage and different stages of convalescence (days 7, 11, and 22) (*, <0.05 to 0.01; **, <0.01 to 0.001; ***, <0.001). The Wilcoxon signed rank test or the Mann-Whitney U test was used for analyses of statistical significance.

of the IgA ASC response was, on average, sixfold lower in O139 cholera patients. It may be mentioned that we were unable to study more O139 cholera patients for ASC responses (only 17 patients with O139 cholera compared to 28 patients with O1 cholera infection) since the incidence of cholera due to this pathogen decreased by the middle of 1994, when the assay was optimized for studying the MSHA-specific ASC responses. The lower ASC response to MSHA in O139 cholera patients correlates with our findings in an earlier study (22), in which the O139 cholera patients showed a significantly lower vibriocidal antibody response than the O1 cholera patients to the homologous target organism. In the present study a difference was also seen in the kinetics of the IgG response in plasma between O1 and O139 cholera patients. More O1 than O139 cholera patients already had elevated levels of anti-MSHA antibodies at the acute stage. The reasons for this may be that the capsule in the O139 organism may prevent the MSHA pili from being optimally exposed and from generating a IgG secondary systemic response as quickly as that in patients with O1 cholera infection. However, there were no differences in the magnitude of the plasma antibody responses between the O1 and O139 cholera patients.

The fecal antibody response, which is probably a more direct measure of the local antibody response in the gut, was similar in both groups of patients, showing that they have comparable local antibody responses. This suggests that the MSHA present on the encapsulated O139 pathogen is able to stimulate the gut lymphocytes as efficiently as the nonencapsulated O1 bacteria. A more direct assessment would be to study the response of lymphocytes isolated directly from the intestinal mucosa for MSHA-specific responses.

To understand the basis for the poorer ASC response in O139 cholera patients, we studied the bacterial strains isolated from the feces of these patients and found that five of the O139 strains were lacking in MSHA activity. However, further studies by immunoblotting and PCR with 3 strains showed that the MSHA subunit was present, as was the gene for the major subunit. The lack of the intact pilus on two of these O139 strains corresponded to the lack of an ASC response and also to the lack of a response of the IgA isotype to MSHA in plasma. Therefore, at least in these five patients it was apparent that strains negative for MSHA activity failed to stimulate a IgA-specific response to MSHA. However, this was not the only explanation since there were four other patients who were infected with MSHA-positive O139 strains but who failed to respond with specific ASCs. It is possible that the other factor may be that the polysaccharide capsule that is present on O139 strains, but that is absent from O1 strains, may in some cases shield the pili. However, O139 cholera patients did respond to MSHA with antibodies in plasma and feces which were not significantly lower than those seen in O1 patients. In addition, it was also observed that two O1 cholera patients infected with MSHA-positive strains also failed to respond, suggesting that the antigen may not always be expressed optimally in vivo. A previous study carried out with serum from cholera patients in Peru has shown that only about 60% of the patients responded with serum antibodies to MSHA at the convalescent stage (29). However, Bangladeshi patients, who, contrary to Peruvians, most likely have been primed (since Bangladesh is an area where cholera is endemic), showed a response early after the onset of the disease and more than 90% of them were positive for antibodies.

Strains of *V. cholerae* O139 have earlier been shown to be positive for MSHA (12, 13, 18, 26). The nucleotide sequence of the *mshA* gene, which encodes the major subunit of MSHA pili, has been found to be identical to the sequence previously

described for an O1 El Tor strain (12). In addition, MAb 17:10 binds along the entire pilus of both *V. cholerae* O1 and an O139 strain 4260B, confirming their similarities (10a). However, it is possible that the pilus in O139 strains may include additional subunits, the distribution of which may vary between O1 and O139 strains or among O139 strains.

The response to bacterial antigens of the IgA isotype in the plasma of patients with enteric infection has been suggested to indicate that an IgA response in the systemic compartment reflects a mucosal response (27, 28). Thus, the presence of MSHA-specific antibodies in the circulation may represent a spillover effect in which excess antibody from the gut enters the systemic compartment. Conversely, the systemic antibodies may diffuse from the circulation into the intestine and possibly protect against disease. However, the presence of specific antibodies of the IgG isotype in plasma indicates that the systemic compartment is also involved in the immune response to the putative adhesion antigen.

In conclusion, this study shows that antibodies to MSHA are produced by patients infected with *V. cholerae* O1 or *V. cholerae* O139. Although the study may not predict the protective role of MSHA-specific antibodies against disease, it shows that in addition to being immunogenic in animals, MSHA is also immunogenic in humans with natural disease. If MSHA is proven to be a colonization factor and a protective antigen, an immune response to it may be used as a correlate of protection.

ACKNOWLEDGMENTS

This research was supported by the Swedish Agency for Research Cooperation with Developing Countries (SAREC), European Union (DG12 HSMU), and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors include the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, China, Denmark, Germany, Japan, The Netherlands, Norway, Republic of Korea, Saudi Arabia, Sri Lanka, Sweden, Switzerland, Thailand, the United Kingdom, and the United States; international organizations including the Arab Gulf Fund, the Asian Development Bank, European Union, the United Nations Children's Fund (UNICEF), the United Nations Development Program (UNDP), the United Nations Population Fund (UNFPA), and the World Health Organization (WHO); private foundations including Aga Khan Foundation, Child Health Foundation, Ford Foundation, Population Council, Rockefeller Foundation, and the Sasakawa Foundation; and private organizations including American Express Bank, Bayer AG, CARE, Family Health International, Helen Keller International, the Johns Hopkins University, Macro International, New England Medical Centre, Procter & Gamble, RAND Corporation, SANDOZ, Swiss Red Cross, the University of Alabama at Birmingham, the University of Iowa, and others.

We thank Manzurul Haque for secretarial assistance.

REFERENCES

1. Åhren C., A. Jertborn, and A.-M. Svennerholm. Unpublished data.
2. Åhren, C., C. Wenneras, 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* 11:929-935.
3. Albert, M. J. 1994. *Vibrio cholerae* O139 Bengal. *J. Clin. Microbiol.* 32:2345-2349.
4. Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruz-zaman, S. M. Faruque, and R. B. Sack. 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet* 341:704. (Letter.)
5. Attridge, S. R., P. Manning, J. Holmgren, and G. Jonson. 1996. Relative significance of the mannose-sensitive hemagglutinin and the toxin-coregulated pili in colonization of infant mice by *Vibrio cholerae* O1 El Tor. *Infect. Immun.* 64:3369-3373.
6. Benenson, A. S., M. R. Islam, and W. B. Greenough III. 1964. Rapid identification of *Vibrio cholerae* by dark field microscopy. *Bull. W. H. O.* 30:827-831.

7. Czerkinsky, C., Z. Moldoveanu, J. Mestecky, L. A. Nilsson, and O. Ouchterlony. 1988. A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells. *J. Immunol. Methods* **115**:31–37.
8. Ferguson, A., K. A. Humphreys, and N. M. Croft. 1995. Technical report: results of immunological tests on faecal extracts are likely to be extremely misleading. *Clin. Exp. Immunol.* **99**:70–75.
9. Franzon, V. L., A. Barker, and A. Manning. 1993. Nucleotide sequence encoding the mannose-fucose-resistant hemagglutinin of *Vibrio cholerae* O1 and construction of a mutant. *Infect. Immun.* **61**:3032–3037.
10. Jertborn, M., A.-M. Svennerholm, and J. Holmgren. 1986. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. *J. Clin. Microbiol.* **24**:203–209.
- 10a. Jonson, G. Unpublished data.
11. Jonson, G., J. Holmgren, and A.-M. Svennerholm. 1991. Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. *Microb. Pathog.* **11**:433–441.
12. Jonson, G., M. Lebens, and J. Holmgren. 1994. Cloning and sequencing of *Vibrio cholerae* mannose-sensitive hemagglutinin pilin gene localization of *mshA* within a cluster of type 4 pilin genes. *Mol. Microbiol.* **13**:109–118.
13. Jonson, G., J. Osek, A.-M. Svennerholm, and J. Holmgren. 1996. Immune mechanisms and protective antigens of *Vibrio cholerae* serogroup O139 as a basis for vaccine development. *Infect. Immun.* **64**:3778–3785.
14. Jonson, G., J. Sanchez, and A.-M. Svennerholm. 1989. Expression and detection of different biotype-associated cell-bound haemagglutinins of *Vibrio cholerae* O1. *J. Gen. Microbiol.* **135**:111–120.
15. Kantele, A. M., T. Takanen, and A. Arvilommi. 1988. Immune response to acute diarrhea seen as circulating antibody-secreting cells. *J. Infect. Dis.* **158**:1011–1016.
16. Monsur, K. A. 1961. A highly selective gelatin-taurocholate tellurite medium for the isolation of *Vibrio cholerae*. *Trans. R. Soc. Trop. Med. Hyg.* **55**:440–442.
17. Mukhopadhyay, S., G. Ghosh, and A. C. Ghose. 1996. Phenotypic expression of a mannose-sensitive hemagglutinin by a *Vibrio cholerae* O1 El Tor strain and evaluation of its role in intestinal adherence and colonization. *FEMS Microbiol. Lett.* **138**:227–232.
18. Nandy, R. K., T. K. Sengupta, S. Mukhopadhyay, and A. C. Ghose. 1995. A comparative study of the properties of *Vibrio cholerae* O139, O1 and other non-O1 strains. *J. Med. Microbiol.* **42**:251–257.
19. Osek, J., G. Jonson, A.-M. Svennerholm, and J. Holmgren. 1994. Role of antibodies against biotype-specific *Vibrio cholerae* pili in protection against experimental classical and El Tor cholera. *Infect. Immun.* **62**:2901–2907.
20. Osek, J., A.-M. Svennerholm, and J. Holmgren. 1992. Protection against *Vibrio cholerae* El Tor infection by specific antibodies against mannose-binding hemagglutinin pili. *Infect. Immun.* **60**:4961–4964.
21. Qadri F., T. Azim, A. Chowdhury, J. Hossain, R. B. Sack, and M. J. Albert. 1994. Production, characterization, and application of monoclonal antibodies to *Vibrio cholerae* O139 synonym Bengal. *Clin. Diagn. Lab. Immunol.* **1**:51–54.
22. Qadri, F., G. Mohi, J. Hossain, T. Azim, A. M. Khan, M. A. Salam, R. B. Sack, M. J. Albert, and A.-M. Svennerholm. 1995. Comparison of the vibriocidal antibody response in cholera due to *Vibrio cholerae* O139 Bengal with the response in cholera due to *Vibrio cholerae* O1. *Clin. Diagn. Lab. Immunol.* **2**:685–688.
23. Quiding, M., I. Nordstrom, A. Kilander, G. Andersson, L.-A. Hanson, J. Holmgren, and C. Czerkinsky. 1991. Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses, gamma-interferon production, and evokes local immunological memory. *J. Clin. Invest.* **88**:143–148.
24. Rahman, M., D. A. Sack, S. Mahmood, and A. Hossain. 1987. Rapid diagnosis of cholera by coagglutination test using 4-h fecal enrichment cultures. *J. Clin. Microbiol.* **25**:2204–2206.
25. Raqib, R., S. Tzipori, M. Islam, and A. A. Lindberg. 1993. Immune responses to *S. dysenteriae* 1 and *S. flexneri* lipopolysaccharide and polysaccharide antigens in Bangladeshi patients with shigellosis. *Serodiagn. Immunother. Infect. Dis.* **1**:37–45.
26. Sack, R. B., and M. J. Albert. 1994. Summary of cholera vaccine workshop. *J. Diarrhoeal Dis. Res.* **12**:138–143.
27. Stoll, B. J., A.-M. Svennerholm, L. Gothefors, D. Barua, S. Huda, and J. Holmgren. 1986. Local and systemic antibody responses to naturally acquired enterotoxigenic *Escherichia coli* diarrhoea in an endemic area. *J. Infect. Dis.* **153**:527–534.
28. Svennerholm, A.-M., M. Jertborn, L. Gothefors, A. M. M. Karim, D. A. Sack, and J. Holmgren. 1984. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. *J. Infect. Dis.* **149**:884–893.
29. Svennerholm, A.-M., G. Jonson, and J. Holmgren. 1994. Immunity to *Vibrio cholerae* infection, p. 257–271. In K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Molecular to global perspectives*. American Society for Microbiology, Washington, D.C.
30. Svennerholm, A.-M., G. Jonson, and C. Yan. 1991. A method for studies of an El Tor-associated antigen of *Vibrio cholerae* O1. *FEMS Microbiol. Lett.* **79**:179–186.
31. Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalonos. 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc. Natl. Acad. Sci. USA* **84**:2833–2837.
32. Thelin, D. L., and R. K. Taylor. 1996. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. *Infect. Immun.* **64**:2853–2856.
33. Voss, E., P. A. Manning, and S. R. Atttridge. 1996. The toxin-coregulated pilus is a colonization factor and protective antigen of *Vibrio cholerae* El Tor. *Microb. Pathog.* **20**:141–153.
34. Wenneras, C., A.-M. Svennerholm, C. Åhren, and C. Czerkinsky. 1992. Antibody-secreting cells in human peripheral blood after oral immunization with an inactivated enterotoxigenic *Escherichia coli* vaccine. *Infect. Immun.* **7**:2605–2611.
35. World Health Organization. 1987. Programme for control of diarrhoeal diseases (CDD/83.3 Rev 1), p. 9–20. In *Manual for laboratory investigations of acute enteric infections*. World Health Organization, Geneva, Switzerland.
36. World Health Organization. 1990. Manual for the treatment of diarrhoea: for use by physicians and other senior healthworkers (CDD/SER/80.2 Rev 2), p. 5–7. World Health Organization, Geneva, Switzerland.