Enterotoxin-Specific Immunoglobulin E Responses in Humans after Infection or Vaccination with Diarrhea-Causing Enteropathogens

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Cholera toxin (CT)-specific antibody responses of the immunoglobulin E (IgE) isotype in the sera of adult patients suffering from infection with either Vibrio cholerae O1, V. cholerae O139, or enterotoxigenic Escherichia coli (ETEC) were analyzed and compared with those in the sera of volunteers immunized with a bivalent B subunit O1/O139 whole-cell cholera vaccine. A significant IgE response to CT was observed in 90% of the patients with V. cholerae O1 infection (18 of 20; P < 0.001) and 95% of the patients with V. cholerae O139 infection (19 of 20; P < 0.001). Similarly, the majority of the patients with ETEC diarrhea (83%; 13 of 15) showed a positive IgE response to CT. Eight of 10 North American volunteers (80%) orally challenged with V. cholerae O1 showed CT-specific IgE responses (P = 0.004). In contrast, Swedish volunteers immunized with the oral cholera vaccine showed no IgE responses to CT (P value not significant). During the study period, total IgE levels in the sera of the diarrheal patients, the North American volunteers, and the Swedish cholera vaccinees alike remained unchanged. However, the total IgE levels in the sera of patients and healthy Bangladeshi controls were on average 89-fold higher than those in the sera of the healthy Swedish volunteers and 34-fold higher than those in the sera of the North American volunteers.

Cholera toxin (CT) is an extensively studied protein enterotoxin, produced by strains of Vibrio cholerae O1 (7) as well as by the more recently described serogroup O139 (4). Patients with cholera seroconvert to CT with antibodies of the immunoglobulin A (IgA) and IgG isotype. Experiments with mice indicated that when CT is administered as a mucosal adjuvant it stimulates a predominantly Th2-type immune response with increased interleukin 4 (IL-4) levels and associated increments in total and specific IgE antibody levels (18, 34). It has been shown that CT affects the release of IL-6 and tumor necrosis factor alpha but not histamine by rat peritoneal mast cells (16). Hitherto, increased levels of IgE antibodies have mainly been described for allergic disorders and parasitic infections, especially intestinal worm infections. However, a recent study demonstrated Haemophilus influenzae and Streptococcus pneumoniae-specific IgE responses in patients with chronic bronchitis (15). Further, immunization against diphtheria and tetanus in adults has been shown to result in specific IgE responses (2). In Helicobacter pylori-infected children, an increase in IgE antibody levels has also been observed (28). Patients infected with Mycobacterium tuberculosis had increased levels of total IgE in sera as well as ascariis-specific IgE responses (1). Whether IgE responses occur in humans exposed to enterotoxin during cholera and other secretory diarrheal diseases is not known. We have therefore investigated whether CT and the Escherichia coli heat-labile enterotoxin are able to induce IgE responses in patients suffering from cholera or diarrhea due to enterotoxigenic E. coli (ETEC). We have, in addition, studied North American volunteers challenged with live V. cholerae O1 and Swedish volunteers orally immunized with the bivalent B subunit O1/O139 whole-cell (B-O1/O139 WC) cholera vaccine and evaluated their CT-specific IgE responses.

For this purpose, 55 adult male Bangladeshi patients with acute watery diarrhea were recruited. Among these, 20 were found to be infected with V. cholerae O139, 20 were found to be infected with V. cholerae O1 El Tor (18 Ogawa and 2 Inaba strains), and 15 were found to be infected with ETEC strains. The patients were 18 to 45 years of age, had a history of 4 to 15 h (median, 8 h) of watery diarrhea prior to hospitalization, and suffered from moderate to severe dehydration. Venous blood was collected from the cholera patients at the acute stage of the disease, i.e., on the second day of hospitalization, which was considered to be approximately 2 days after the onset of diarrhea for the purpose of this study (day 2). Blood was also collected 5, 9, and 20 days later, during convalescence (that is 7; 11, and 22 days after the onset of diarrhea, respectively). From the 15 patients with ETEC diarrhea, samples could only be collected at the acute stage of infection (day 3 after the onset of diarrhea) and about 6 days later, at convalescence (day 9 after the onset of diarrhea); late-convalescence-stage samples could not be collected. Sera were separated from blood samples and stored in aliquots at −20°C until tested. Feces samples were also collected on each study day, and fecal extracts were prepared and stored in aliquots at −70°C (21). Sera from 10 adult North American volunteers orally challenged with 105 CFU of live V. cholerae O1, El Tor Inaba (24) were also analyzed. Samples collected prior to challenge (day 0) and 7, 10, and 14 days after challenge were tested. Sera from 20 Swedish volunteers orally immunized with two
doses of the B-O1/O139 WC cholera vaccine were also analyzed in the study (12). Serum samples were collected prior to immunization (day 0) and around 7 days after intake of two doses of the vaccine (day 21 or 22). Twenty-six adult males of similar age as the patients (i.e., 18 to 40 years of age) and of similar socioeconomic background, but with no history of diarrhea during the previous 6 months, were included as controls and are referred to herein as Bangladeshi controls. The preimmunization (day 0) samples from North American and Swedish volunteers were considered control specimens. Informed consent was obtained from the patients and controls.

The study was approved by the ethical review committees of the respective institutions.

Microbiological confirmation of strains was carried out using standard procedures as described earlier for *V. cholerae* O1 and O139 (22) and ETEC (32). All 15 ETEC strains produced both heat-labile and heat-stable enterotoxins (30, 31). Slide agglutination with specific monoclonal antibodies (17) further showed that all ETEC strains produced defined colonization factor (CF) antigens (6). One strain was positive for CFA/I, four strains were positive for CFA/II, and 10 strains were positive for CFA/IV.

The total IgE content in serum samples was determined by an enzyme-linked immunosorbent assay (ELISA) method (18) as follows. Wells of microtiter plates (Nunc Immuno, Roskilde, Denmark) were coated with 100 µl of goat anti-human IgE (1:1,000 dilution; Sigma, St. Louis, Mo.) in 10 mM phosphate-buffered saline (PBS), pH 7.2, and incubated overnight at 4°C. After being washed three times with PBS, wells were blocked with 10% goat serum (200 µl per well; Sigma) for 1 h at room temperature. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween; Sigma) and then 100 µl of human standard IgE myeloma (starting concentration, 1 µg/ml; Calbiochem, La Jolla, Calif.) or sera from patients or controls was added to wells in duplicate and serial dilutions (in PBS-Tween) were carried out. The serum samples were serially diluted twofold starting at 1:50 for cholera patients and Bangladeshi controls and at 1:5 for non-Bangladeshi individuals. Incubation was carried out for 4 h at 37°C. After five washes with PBS-Tween, 100 µl of biotinylated mouse anti-human IgE (1:2,000 dilution; Sigma) was added and the plates were incubated overnight at 4°C. After five washes with PBS-Tween, streptavidin-conjugated horseradish peroxidase (100 µl per well, 1:4,000 dilution in PBS-Tween; Amersham, Buckinghamshire, United Kingdom) was added. Plates were incubated for 90 min at room temperature. After five washes with PBS-Tween the substrate 3,3',5,5'-tetramethyl benzidine (Sigma) was added, and after 30 min, the color development was stopped by the addition of 1.0 M H2SO4 (50 µl/well) and absorbance was measured at 450 nm. Total IgE levels in serum samples were quantified (in micrograms per milliliter) by comparison with the IgE myeloma protein standard. IgE levels in fecal extracts prepared from stools (21) obtained from eight *V. cholerae* O1- and eight *V. cholerae* O139-infected patients (at acute stage, on day 2, and during convalescent stage, on day 22) were also determined.

Serum samples were tested for CT-specific antibodies of the IgE isotype (18) using a modified version of the previously described GM1 ELISA method (29) with the recombinant B-subunit of the cholera toxin (rCTB) as the coating antigen (25). Microtiter plates (Nunc Immuno) were coated with 100 µl of GM1 (Sigma) per well in PBS (1 mmol/ml) and incubated overnight at room temperature. After three washes with PBS, rCTB was added (100 µl/well, 2 µg/ml) and the plates were incubated at room temperature for 1 h. After three washes with PBS, blocking was carried out with 10% goat serum (200 µl per well) for 1 h at room temperature. After five washes with PBS-Tween, samples were applied at an initial dilution of 1:2 and then serial twofold dilutions were carried out. Plates were incubated for 4 h at 37°C. After five washes with PBS-Tween, biotinylated mouse anti-human IgE (1:500 dilution in PBS-Tween; Pharmingen) was added and the plates were incubated overnight at 4°C. After five washes, streptavidin-conjugated horse radish peroxidase (1:500 dilution in PBS-Tween) was added, the color reaction developed as described above, and endpoint titers were determined (21). A cholera patient, challenged volunteer, or vaccinee showing a twofold or greater increase in CT-specific IgE antibody response over the acute-stage level at any point during the convalescent stage was designated a responder. To confirm that the IgE anti-CT response observed in the cholera and ETEC-infected patients was specific, we assayed patient sera for responsiveness to an unrelated protein of parasite origin, namely, *Entamoeba histolytica* (extracts of axenic culture of strain HM1:1MSS). An ELISA procedure described earlier was employed (19, 20) using the protein extract (10 µg/ml) as the coating antigen. The remaining procedures were similar to those described above for detecting the CT-specific IgE responses.

For statistical analyses of results, the Wilcoxon signed-rank test and the Mann-Whitney U test were used where applicable; a *P* value of <0.05 was the criterion for a significant difference. Geometric means (GM) and the standard deviations or the standard errors of the means (SEM) were calculated for samples.

The concentration of total IgE in the sera of cholera and ETEC-infected patients from Bangladesh varied from about 0.2 µg/ml to a maximum of over 70 µg/ml. No increase in total IgE levels in sera was observed between the acute and convalescent stages of the disease in *V. cholerae* O1-, *V. cholerae* O139-, or ETEC-infected patients (*P* value was not significant [NS] in all cases) (Table 1). Furthermore, the total IgE levels in patients were not different from those seen in Bangladeshi controls. The total IgE levels in patients and healthy Bangladeshi controls were on average 34 times higher than those in the North American individuals and 89-fold higher than those in the Swedish individuals. However, as with the Bangladeshi, there were no increases (*P* value was NS) in the levels of total IgE after oral challenge or immunization in either North Americans or Swedes. In contrast, the levels of total IgE in the sera of North American volunteers and the Swedish vaccinees (Table 1) were significantly lower than those in the healthy Bangladeshi controls and in the patients (*P* = 0.001). No IgE could be detected in the fecal extracts of the cholera patients.

Our findings corroborate earlier studies in developing countries which have shown that the sera of both healthy individuals and subjects with symptomatic as well as asymptomatic parasitic infections have elevated levels of IgE compared to individuals residing in developing countries (1, 8, 13, 20). Since there is an association between helminthic infections and elevated levels of IgE and the parasite prevalence rate is high in healthy subjects in developing countries (14), we examined our patients for the presence of common parasites in the stool both at the acute stage and during convalescence. Stools were examined by direct microscopy to detect cyst and vegetative forms of parasites and ova of helminths. Neither helminths nor other common parasites were detected in the stools of the 40 cholera patients. Among the ETEC-infected patients, however, three had cysts of *Giardia lamblia* in their stool samples. In examinations of the stool samples from Bangladeshi controls, *Trichuris trichiura* was detected in two samples, *Ascaris lumbricoides* was detected in two samples, and hookworm was detected in their stool samples.
detected in one sample. Thus, in spite of the presence of *G. lamblia* in 3 of 15 ETEC-infected patients and the presence of other parasites in 5 of the 26 Bangladeshi controls, more than 90% of the Bangladeshi study subjects were found to be free of parasites; however, these persons may have harbored parasites a short time prior to the start of the study.

Elevated levels of IgE can also result from the presence of blood-borne parasites. For this reason, stained blood smears were also screened for the presence of malarial and filarial parasites (33). However, these parasites were not detected in the blood of either patients or healthy Bangladeshi controls.

The majority of the cholera patients (18 of 20 infected with *O1* serogroup strains and 19 of 20 infected with *O139* serogroup strains) had significantly increased titers of IgE specific for CT in serum by day 22, i.e., 3 weeks after the onset of diarrhea (Fig. 1). The CT-specific IgE antibody response was very rapid, a fact reflected by the finding that a significant response was observed in more than half of the patients by day 7 after the onset of illness. The IgE response to CT in the cholera patients showed a wide variation (titer range, 1.1 to >209 arbitrary units) (Fig. 1). The CT-specific IgE response was also analyzed in relation to the total serum IgE concentration (specific IgE/total IgE in samples) in order to correct for differences in individual total IgE concentrations. The same responder frequency was observed using these calculations; significant CT-specific responses were found between the acute (day 2) and convalescent (day 7, 11 or 22) phases of infection in both *O1* (*P* = 0.001) and *O139* (*P* = 0.001) cholera patients. The maximal average increase in CT-specific IgE antibody titer was 11-fold on day 11 for *O139* patients and 10-fold on day 22 for *O1* patients compared to the acute-stage levels. No CT-specific IgE response could be detected in the fecal extracts obtained from any of the cholera patients.

For the ETEC-infected patients, a CT-specific IgE titer 2 standard deviations above the GM level recorded in controls, i.e., a CT-specific IgE titer of >1.00, was taken as a positive response. The reason for this was that serum could only be collected later after the onset of diarrhea (day 3 after hospitalization), when the CT-specific IgE levels were already elevated (Fig. 1). Of the 15 patients with ETEC diarrhea, 13 responded with IgE specific to CT at the acute phase (day 3), and of the 13 patients evaluated at the convalescent phase of infection, 11 showed a positive response. However, no significant difference in response was found between the acute and convalescent phases of infection (*P* = 0.547). This is probably because the acute- and the convalescent-stage samples were collected too early after the onset of diarrhea. A late-convalescent-stage sample (e.g., 3 weeks later) could not be collected as for the cholera patients. The response to CT at the acute stage of ETEC infection was more elevated than the response seen at the acute stage (day 2) in *V. cholerae* O1-infected (*P* = <0.001) and *V. cholerae* O139-infected (*P* = 0.002) patients.

The North American volunteers showed significantly increased CT-specific IgE responses by day 10 (5 of 10 responders, *P* = 0.01) and day 14 (8 of 10 responders, *P* = 0.004) after challenge with *V. cholerae* O1 (Fig. 1). The response on day 14 after challenge was in fact higher in the North American volunteers than the response recorded on day 22 after onset of illness in Bangladeshi patients with natural *V. cholerae* O1 infection (*P* = 0.037) but not that in patients with *V. cholerae* O139 infection. In contrast, no significant increase in CT-specific IgE antibody titer was detected in the 20 Swedish vaccinees after immunization with the killed oral cholera vaccine (*P* value was NS). Only two vaccinees showed a marginal increase in antibody titers after immunization with two doses of

### Table 1. Total IgE antibody titers in serum

<table>
<thead>
<tr>
<th>Study group</th>
<th>Total IgE (µg/ml) on days*</th>
<th>0–3</th>
<th>7–9</th>
<th>10–11</th>
<th>14–22</th>
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<tbody>
<tr>
<td><em>V. cholerae</em> O1-infected patients</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GM</td>
<td>4.11</td>
<td>4.72</td>
<td>4.18</td>
<td>5.46</td>
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<tr>
<td>Range</td>
<td>2.08–5.68</td>
<td>3.53–6.31</td>
<td>3.17–5.51</td>
<td>3.94–7.55</td>
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<td>20</td>
<td>20</td>
<td>20</td>
<td></td>
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<tr>
<td><em>V. cholerae</em> O139-infected patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>2.64</td>
<td>2.17</td>
<td>4.04</td>
<td>2.30</td>
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<tr>
<td>Range</td>
<td>1.89–3.70</td>
<td>1.69–2.80</td>
<td>3.09–5.37</td>
<td>1.74–3.05</td>
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<tr>
<td><em>n</em></td>
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<td>20</td>
<td>20</td>
<td>20</td>
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<tr>
<td>ETEC-infected patients</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>GM</td>
<td>5.0</td>
<td>5.02</td>
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<td></td>
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<tr>
<td>Range</td>
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<td>3.99–6.32</td>
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<tr>
<td><em>n</em></td>
<td>15</td>
<td>13</td>
<td></td>
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<tr>
<td>North American volunteers challenged with <em>V. cholerae</em> O1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GM</td>
<td>0.13</td>
<td>0.13</td>
<td>0.15</td>
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<tr>
<td>Range</td>
<td>0.08–0.20</td>
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<td>0.08–0.25</td>
<td>0.08–0.22</td>
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<tr>
<td><em>n</em></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Swedish cholera vaccinees</td>
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<tr>
<td>GM</td>
<td>0.05</td>
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<tr>
<td>Range</td>
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<td>20</td>
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</table>

*Days after onset of diarrhea or after oral challenge with live *V. cholerae* O1 or immunization with the bivalent B-O1/O139 WC cholera vaccine. Day 0 levels are preimmune levels.*

*b* Range is GM of IgE level ± 1 SEM.

*n* = number of study persons. The GM titer of total IgE in sera of healthy Bangladeshi controls (*n* = 26) was 4.45 µg/ml, and the range was 3.32 to 5.6 µg/ml.
the cholera vaccine. The same volunteers developed IgA and IgG antibody responses to the toxin (12).

To confirm that the IgE anti-CT response observed in the cholera and ETEC-infected patients was specific, we assayed patient sera for responsiveness to an unrelated protein of parasitic origin, namely, *E. histolytica*. Entamoeba infections are prevalent in populations of low socioeconomic status in the developing world and give rise to strong IgE responses in serum. None of the patients or controls in the study were infected, however. The diarrheal patients had low antibody titers to this antigen at both the acute (GM titer range, 0.35 to 0.47) and the convalescent phases of infection (GM titer range, 0.44 to 0.66). In summary, neither the patients nor the controls had significant responses to *E. histolytica*.

To further study the specificity of the CT-IgE response, we also tested serum samples from patients with an unrelated invasive diarrheal disease, shigellosis. Fourteen pairs of serum samples (23) from patients with either *Shigella dysenteriae* type 1 (n = 10) or *Shigella flexneri* (n = 4) infections were tested in the ELISA for responses to CT. None of the patients showed any serum antibody response to CT of the IgE isotype.

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