Antibody Responses to *Escherichia coli* O157 and Other Lipopolysaccharides in Healthy Children and Adults

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In Mexico, diarrheal disease due to different serotypes of *Escherichia coli* is highly prevalent, with only sporadic isolation of O157 non-H7 strains. This could be due to exposure to the O157 or related *E. coli* lipopolysaccharide (LPS), such as O7 or O116, at an early age. By using enzyme-linked immunosorbent assay (ELISA) and Western blotting, the present study analyzed 605 serum samples from Mexican adults and infants without clinical symptoms of disease for the presence of antibodies to these three *E. coli* LPSs. The bactericidal activities of homologous and heterologous rabbit and human serum samples against O7, O116, and O157 *E. coli* LPSs were also determined. By using a cutoff point of 0.7, it was found by the ELISAs that 28 of 562 (5%) of the serum samples from adolescents and adults and 2 of 43 (5%) of the serum samples from infants less than 1 year of age reacted with the O157 LPS. By using cutoff points between 0.4 and 0.699, the proportion of serum samples from both age groups that reacted with the O157 LPS increased to 20%. Western blotting analysis of selected serum samples that showed an intermediate response against the O157 LPS by the ELISAs showed that 61 of 88 (69%) reacted with the same LPS. A similar result was observed for maternal milk samples. The bactericidal activities of rabbit serum samples against the O7, O116, and O157 LPSs showed that they were positive for both homologous and heterologous antigens. Similar results were observed with the human serum samples. O157 non-H7 strains were identified in only 10% of the *E. coli* strains isolated from 263 Mexican children with and without diarrhea over the past 15 years. This absence of O157:H7 strains in Mexico may be associated with the presence of antibodies against O157 or related *E. coli* LPSs.

Enterohemorrhagic *Escherichia coli* O157:H7 is of great clinical and epidemiological importance as a cause of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in developed countries (2, 21, 35). The incidence of these bacteria in developing countries (2, 21, 35). The incidence of these bacteria in Mexico and other developing countries is low, although it has been reported that these bacteria are present in Chile, Brazil, Argentina, and Colombia (12, 17, 19, 20).

The presence of antibodies in sera from patients with HUS against the lipopolysaccharide (LPS) of *E. coli* O157 was reported initially by Notenboom et al. (23). Subsequent studies designed to characterize the immune responses of patients infected with *E. coli* O157 have reported the presence of immunoglobulin M (IgM) (8), IgG (16), and both IgM and IgG (1) antibodies to O157 LPS in patients with HUS. IgA antibodies have also been reported in the serum and feces of patients with HUS (6, 30). A study carried out by Reymond et al. (28) showed the presence of antibodies to O157 LPS in 12% of the population living in rural areas where O157 strains are endemic, whereas they were found in only 4.7% of the population living in urban areas. Similar results were observed by Belongia et al. (3) in a rural Wisconsin population.

Cross-reactivities between the LPS of *E. coli* O157 and O antigens of *Yersinia enterocolitica* O9, *Citrobacter freundii*, *Escherichia hermannii*, and *Brucella abortus* (5, 9, 29, 31) have been reported previously. It has also been reported that the serum of patients with HUS associated with *E. coli* O157 reacted with strains of *Vibrio cholerae* O1 Inaba. In another study with serum from individuals vaccinated against *V. cholerae* O1 (7), the sera were seen to react strongly against O157 LPS. Similarly, cross-reactivities between the *E. coli* O157 serogroup and the *E. coli* O7 and O116 serogroups have been described (15).

In Mexico, there have been no reports of HUS or HC in association with *E. coli* O157:H7 infection. The reason for this lack of infection has not been explained. The aim of this study was to determine the humoral response against *E. coli* O157 LPS and cross-reacting O7 and O116 LPSs in healthy Mexican infants and adults and the possible participation of this cross-reactivity as an important factor preventing colonization and disease associated with *E. coli* serogroup O157 infection.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* strains of serogroups O7:NM (nonmotile), O116:H10, O157:H19, and O157:H7 (provided by B. Rowe, Department of Enteric Pathogens, Central Public Health Laboratory, London, England) were used for the different assays.

*E. coli* O7, O116, and O157 in Mexican children. The frequency of isolation of *E. coli* strains of serogroups O7, O116, and O157 in different studies conducted from 1985 to 1987 (13) and between 1996 and 2000 (unpublished data) was used as a reference to analyze the results presented in this study. The isolation and microbiological characteristics of these strains were determined as described previously (13).

**Serum samples.** The 605 serum samples used in this study were obtained by the Regional Public Health Laboratory of Hermosillo, Sonora, Mexico, from 562 adolescents and adults (age range, 15 to 40 years) without any disease symptoms and from 43 asymptomatic infants (age, less than 1 year) in Mexico City who had
been used as controls in several immunization studies (Regional Public Health Laboratory serum bank). Written informed consent was obtained from the adults or the care providers of the minors in every case.

**Breast milk samples.** Seven breast milk samples from Mexican women (14) were evaluated for their reactivities with the E. coli O157 LPS.

**Rabbit antiserum.** Antisera against E. coli O7:NM, O116:H10, and O157:H19 strains were prepared in rabbits by the procedures detailed by Ewing (15).

**LPS purification.** Bacterial LPS was extracted by the phenol-water method described by Westphal and Jann (36). The LPS obtained was treated with DNase, RNase, and proteinase K. The LPS was lyophilized and kept at room temperature until use. The protein concentration was determined by the method of Bradford (4).

**ELISA.** IgG antibodies against O7, O116, and O157 LPSs were detected by the enzyme-linked immunosorbent assay (ELISA) method described by Chart et al. (8). In brief, ELISA plates were coated with 1 μg of each LPS in 100 μl of coating buffer (1.59 g of NaCO₃ and 2.93 g of NaCHO₃ per liter [pH 9.6]). After the plates were washed in phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Tween 20 (PBS-Tween), unlabelled protein-binding sites were blocked by adding 200 μl of 1% (wt/vol) bovine serum albumin in PBS-Tween to each well (30 min, 37°C). The serum samples (10⁻³) were added to the ELISA plates (100 μl per well) prior to incubation at room temperature for 2 h. The plates were then rinsed with PBS-Tween. To generate the reaction, 100 μl of goat anti-human IgG labeled with alkaline phosphatase (Zymed Laboratories, San Francisco, Calif.) at a dilution of 10⁻⁵ was added to each well, and the plates were incubated at room temperature for 2 h. To visualize the reaction, the plates were washed as described above. Then, 200 μl of p-nitrophenyl phosphate (1 mg/ml; Sigma) in diethanolamine buffer (pH 9.8; Sigma) was added, and the plates were incubated at room temperature for 30 min. The reaction was stopped by adding 25 μl of 3 M NaOH. The optical density values at 405 nm were measured in a microplate ELISA reader (MR 580; Dynatech). For each serum sample, mean readings of the optical density at 405 nm were calculated for the test and the control wells.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of O157 LPS was carried out as described by Laemmli (18) with a 4.5% concentrator gel and 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein loading dye. The LPS puriﬁcation was free of NaCHO₃, and the protein concentration was determined by the method of Bradford (4).

**Western blotting.** Western blotting analysis of the O157 LPS with 88 serum samples and 7 breast milk samples was performed by the method described by Towbin et al. (32). In brief, polyacrylamide gels were transferred to nitrocellulose sheets (Immobilion-NC transfer membranes; Millipore) at 100 μA over 16 h. The sheets were blocked with 5% skim milk in Tris-HCl-buffered saline (TBS; pH 7.4). They were subsequently incubated with the different serum samples diluted 1:50 in TBS-Tween (PBS-Tween), unlabelled protein-binding sites were blocked by adding 200 μl of 1% (wt/vol) bovine serum albumin in PBS-Tween to each well (30 min, 37°C). The serum samples (10⁻³) were added to the ELISA plates (100 μl per well) prior to incubation at room temperature for a further 2 h. The plates were then rinsed with PBS-Tween. To generate the reaction, 100 μl of goat antibody against human IgG labeled with alkaline phosphatase (Zymed Laboratories) was added to each well, and the plates were incubated at room temperature for 4 h. The reaction was incubated at 37°C for 4 h. Suspensions from these cultures containing 1.5×10⁸ CFU/ml were added to 96-well microplates (Difco); and the plates were incubated at 37°C for 18 h. Samples of these cultures were incubated with goat anti-rabbit IgG labeled with alkaline phosphatase (Zymed Laboratories). In all cases, the reaction was visualized with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

**Bactericidal activity of serum.** The bactericidal activities of the serum samples against E. coli O7, O116, and O157 were determined by the method described by Qadri et al. (27).

**RESULTS**

**Frequency of isolation of E. coli serogroups O7, O116, and O157.** Previous studies performed from 1985 to 1987 (13) and between 1996 and 2000 (unpublished data) identified E. coli strains of the O7 serogroup in 31 (12%) of 263 children studied; 23 (74%) of these 31 children had diarrhea. E. coli strains of the O157 serogroup were identified in 26 (10%) of 263 children, and 17 (65%) of these 26 children had diarrhea. E. coli strains belonging to the O116 serogroup were never isolated in the studies.

**ELISA.** In this study a cutoff point of 0.7 was used as a positive value for the ELISAs conducted with the 605 serum samples at a 10⁻³ dilution, as described above. This value corresponded to the mean value (0.350) plus two standard deviations (0.175) when the assays were read at 405 nm. The results showed that 62 (11%) of the 562 serum samples from asymptomatic Mexican adolescents and adults reacted with one of the three LPSs analyzed (O7, O116, and O157). Twenty-eight (5%) of the 562 serum samples from adolescents and adults and 2 (5%) serum samples from the 43 children reacted only with the O157 LPS (Table 1). When cutoff points from 0.4 to 0.699, as proposed by Chart et al. (8), were applied to the ELISA, the results showed that 20% of the serum samples from adolescents and adults had a positive response to the O157 LPS (Table 2).

**Western blotting.** Western blotting analysis of the serum samples with a positive ELISA result (by use of a cutoff point of >0.7) showed that 86% (24 of 28) reacted with the O157 LPS. Similar results were observed for 61 of 88 (69%) of the serum samples with an intermediate ELISA response (by use of cutoff points of 0.4 to 0.699). These reactions were mainly

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**TABLE 1. Responses of human serum samples to different E. coli LPSs determined by ELISA with a cutoff point of 0.7**

| Serum sample source | No. (%) of samples reactive against the following E. coli LPSs: |
|---------------------|---------------------------------------------------------------|
|                     | O7 | O116 | O157 |
| Adults (562)        | 7 (1.0) | 27 (5.0) | 28 (5.0) |
| Children (43)       | 1 (2.0) | 5 (12.0) | 2 (5.0) |
| Total (605)         | 8 (1.0) | 32 (5.0) | 30 (5.0) |

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**TABLE 2. Responses of human serum samples to different E. coli LPSs determined by ELISA with cutoff points of 0.4 to 0.699**

| Serum samples source | No. (%) of samples reactive with the following E. coli LPS: |
|---------------------|---------------------------------------------------------------|
|                     | O7 | O116 | O157 |
| Adults (562)        | 51 (9.0) | 125 (22.0) | 114 (20.0) |
| Children (43)       | 2 (5.0) | 20 (47.0) | 9 (21.0) |
| Total (605)         | 53 (9.0) | 145 (24.0) | 123 (20.0) |
against polysaccharide repeating units and lipid A of the LPS of the E. coli O157 LPS by Western blotting of human serum samples when they were analyzed by the same procedures. The relationship between exposure to microorganisms that share epitopes and disease prevention has been analyzed previously. It has been proposed that the 4-amino-4,6-dideoxy-a-D-mannopyranosyl polysaccharide present in the repeated units of the LPS side chain could be an antigenic determinant related to the immune response (22, 26).

Bactericidal activity of rabbit serum. The rabbit antiserum samples prepared to have activity against O157 LPS were positive for bactericidal activity against O7, O116, and O157 strains. Similar results were observed when anti-O7 and anti-O116 rabbit sera were used (Fig. 3). Significant differences (P < 0.05) between immune and preimmune rabbit antiserum were detected by a one-tailed statistical analysis of variance (Table 3). Similar results were obtained with the three human serum samples tested. These serum samples showed that bactericidal activity was principally against E. coli O157:H7 with serum dilutions of 1:16 and 1:64 (P < 0.05).

DISCUSSION

HC and HUS associated with E. coli O157:H7 infections in developed countries continue to be important public health problems (1, 37). In developing countries, the incidence of these diseases remains low, with only a few cases described so far (12, 17, 19, 20). Despite active surveillance, previous studies in Mexico [A. Navarro, J. L. Mendez, L. M. Perea, C. Eslava, and A. Cravioto, Abstr. VTEC ’97, 3rd Int. Symp. Workshop Shiga Toxin (Verocytotoxin)-Producing Escherichia coli Infections, abstr. V168/IV, p. 77, 1997] have shown that E. coli O157 isolates are rarely isolated and identified.

It has been shown (15, 25) that rabbit antisera prepared against E. coli O7, O116, and O157 LPSs had heterologous reactivities against each other. This observation has been confirmed in our laboratory (Navarro et al., Abstr. VTEC ’97, 3rd Int. Symp. Workshop Shiga Toxin (Verocytotoxin)-Producing Escherichia coli Infections, 1997). The present work extends these observations to human serum samples from individuals in different age groups in Mexico by more quantitative techniques. By the ELISA, 20% of the serum samples studied reacted to the O157, O7, and O116 LPSs. Different studies (3, 28, 38) showed that 12 to 14% of the serum samples obtained from the rural population were positive for reactivity to the O157 LPS. These results suggest the production of an immune response against the O157 LPS induced by contact with common epitopes shared by these bacteria (22, 24, 33).

The relationship between exposure to microorganisms that share epitopes and disease prevention has been analyzed previously. It has been proposed that the 4-amino-4,6-dideoxy-a-D-mannopyranosyl polysaccharide present in the repeated units of the LPS side chain could be an antigenic determinant related to the immune response (22, 26).

In Mexico, contact at an early age with bacteria belonging to the O7 and O116 serogroups could provide antigenic cross-reactivity against E. coli O157 LPS that could protect the individual against subsequent infection with the latter organisms.

Different studies on the incidence of the more common E. coli serotypes conducted in our laboratory from 1985 to 2000 detected only O157 nonmotile or non-H7 strains in 10% of 263 children. A nonspecific response related to the antigenic cross-reaction observed in the study or specific immunity associated with infection with O157 nonmotile strains or O157 non-H7 strains could explain the low incidence of E. coli O157:H7 infections observed in Mexico. Secretory immunoglobulin A (sIgA) is a primary factor responsible for prevention of the attachment of enteric pathogens to the gut epithelium in breast-feeding infants. In this study, anti-O157 LPS antibodies were observed in serum samples from healthy children less than 1 year of age. The anti-O157 antibodies identified in these

FIG. 1. Immune responses of human serum samples against the E. coli O157 LPS by Western blotting of E. coli O157 LPS developed with rabbit and human serum samples. Lanes: 1, molecular size markers; 2, nonimmune rabbit serum (negative reaction); 3, anti-O157 rabbit serum (positive reaction); 4 to 6, human serum samples that showed an intermediate response by ELISA (positive reaction); 7 and 8, human serum samples positive by ELISA (positive reaction); 9, a human serum sample negative by ELISA (negative reaction). The recognition of polysaccharide repeating units of the LPS (ladder pattern), independent of its intensity, was considered a positive reaction. The numbers on the left are in kilodaltons.

FIG. 2. Immune responses of human breast milk samples against E. coli O157 LPS by Western blotting of E. coli O157 LPS developed with breast milk samples from mothers of children less than 1 year of age. Lanes: 1, molecular size markers; 2 to 6, human breast milk samples that reacted with the E. coli O157 LPS. The numbers on the left are in kilodaltons.
children could have been transferred during pregnancy or through breast-feeding. The study identified the existence of sIgA against the O157 LPS in five of seven (71%) of the breast milk samples. Contact by the mothers with E. coli strains of this serotype or with E. coli strains with cross-reacting LPS antigens capable of inducing a heterologous immune response could be associated with the presence of sIgA anti-O157, which thereby provides to the children protection against colonization with these organisms in the first months of life (14).

The neutralizing capacity of serum antibodies is important for the host in the elimination of microorganisms from mucosal surfaces. The results of this study, which showed the heterologous bactericidal activities of rabbit antisera prepared against the O7, O116, and O157 LPSs, suggest that the low incidence of colonization with E. coli O157 and, probably, the low incidence of HUS in regions where diarrheal diseases are considered endemic could be related to the presence of antibodies against these bacteria. Viret et al. (34) showed that immunization of individuals with attenuated vaccines against Salmonella enterica subsp. enterica serovar Typhi and V. cholerae induced antibodies with bactericidal activities against these same bacteria. The antibodies interfered with their capacity to colonize the intestine.

Studies with animal models immunized orally with a strain of S. enterica subsp. enterica serovar Landau, which shares epitopes with E. coli O157 (11), showed the presence of high antibody titers against the O157 LPS. When the animals were orally challenged with a strain of E. coli O157:H7, they showed greater resistance than nonimmunized animals to colonization with these bacteria. In another study with BALB/c mice inoculated orally with E. coli O157:H7 on two occasions, there was a reduction in the period of excretion of the microorganism after the second challenge (10). The resistance to colonization observed showed a correlation to the presence of antibodies to the O157 LPS in the serum and feces of these animals.

As a result of the findings of this study, it would be interesting to see if children with or without E. coli infection had antibodies or to predict the antibody titers for the population from the available data. To answer these questions, the study would have to have a case-control design, which was not the case in this initial exploratory investigation. Since we did not collect serum samples in the studies conducted between 1985 and 1987 and 1996 and 2000, the results of this study and that of a case-control study could be comparable, although this is highly speculative but not unreasonable, given the etiologic data that we have for these children. Recently, Belongia et al. (3) evaluated the presence of anti-O157 antibodies in farm and

| Serotype     | Preimmune | O7  | O116 | O157 |
|--------------|-----------|-----|------|------|
| O7:NM        | 75.0      | 9.0 | 8.4  | 3.8  |
| O116:NM      | 67.5      | 8.7 | 59.5 | 5.5  |
| O157:H7      | 70.5      | 6.2 | 8.7  | 8.7  |
| O157:H19     | 70.5      | 12.7| 46.6 | 7.7  |

The values were for the last serum dilution showing bacterial activity with a statistically significant difference (P < 0.05) when the results obtained were compared with those observed with preimmune serum.
nonfarm residents who made clinical visits due to diarrhea in a rural setting. The results showed that 14% of 363 children had anti-O157 antibodies. Although the study showed that the incidence of clinically recognized diarrhea was similar among children who had and without anti-O157 antibodies, the clinical visit rate due to diarrhea was 46% lower among children who were farm residents. These observations suggest that the reduced occurrence of clinical illness could be associated with repeated antigenic stimulation in a contaminated environment.

In the present study, the presence of antibodies to the O157 LPS in the general population of a developing country like Mexico suggests that the limited isolation of E. coli O157 and the low number of HUS cases in these areas could be related to speciﬁc or nonspeciﬁc protection against a different external antigen that develops during the ﬁrst months of life.

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