Contact Hemolysin Production by Strains of Enteraggregative Escherichia coli Isolated from Children with Diarrhea

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Forty-five strains of enteraggregative Escherichia coli were tested for hemolytic activity with different culture media and erythrocytes from different species. Thirty-seven strains showed proteinase K-sensitive contact hemolysin activity with sheep erythrocytes when cultured in Casamino Acids-yeast extract broth supplemented with 1 mM calcium chloride and when cultured in nutrient broth media. The production of contact hemolysin was dependent on temperature, pH, and culture conditions.

Strains of enteraggregative Escherichia coli (EAggEC) have been implicated as etiologic agents of gastroenteritis in infants in developing countries (4, 6, 10), most prominently among cases of infection which persist longer than 14 days. EAggEC strains are characterized by their distinctive aggregative adherence to HEp-2 or HeLa cells in culture (10, 14). Organisms that demonstrate aggregative adherence have been associated with diarrheal disease (8, 10, 15), and little is known about these non-enteropathogenic E. coli adherent organisms and the development of diarrheal disease (4, 5). The distinct aggregative adherence is also associated with the presence of a 60-MDa plasmid that encodes bundle-forming fibriae 2 to 3 nm in diameter (9). Recently, a plasmid-mediated heat-stable enterotoxin and a heat-labile protein antigenically related to the E. coli hemolysin produced by EAggEC have been reported (2, 12). It has also been reported that EAggEC strains produce hemorrhagic lesions in the rabbit and rat ileal loops, and the hemorrhagic nature of the lesions is consistent with the fact that many children infected with EAggEC experience bloody diarrhea (4, 14).

In this study, 41 strains of EAggEC (1) that showed clump formation at the surface of liquid culture were tested for contact hemolysin (CH) activity with guinea pig, rabbit, rat, sheep, and human type O erythrocytes under different culture conditions. Another four strains, PE 7, PE 42, PE 457, and PE 464 (serotypes O126:H27, O131:H25, O7:H4, and O126:H27, respectively) of EAggEC isolated from infants with diarrhea in Brazil (13) and an avirulent E. coli strain, 36000 (7), were also used for the investigation. All of the EAggEC strains showed aggregative adherence to HeLa cells and positive hybridization with the 32P-labeled EAggEC probe (13, 15). Strains were also identified as EAggEC by clump formation at the surface of the liquid culture at the end of the incubation, as we have described previously (1).

Strains stored at −80°C were subcultured on Trypticase soy agar (Eiken Chemical Co., Tokyo, Japan) containing 0.6% yeast extract (Difco Laboratories, Detroit, Mich.) and grown in screw-cap test tubes containing 10-ml portions of different broth media. Bacteria grown in brain heart infusion broth (Eiken Chemical), Casamino Acids-yeast extract (CYE) broth (Difco), Luria-Bertani (LB) broth (Eiken Chemical), Mueller-Hinton broth (Difco), nutrient broth (Eiken Chemical), and Trypticase soy broth (Difco) at 20 to 42°C for 8 to 24 h with rotation (100 rpm) or statically were used for the experiment. The CH activity of the strains was tested by the modified method of Sansonetti et al. (11). To study CH production by EAggEC, bacteria were washed twice and suspended in phosphate-buffered saline (PBS) at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan) to a concentration of 2 × 1010 CFU/ml. Blood was collected in Alsever’s solution and washed twice, and the erythrocyte concentration was adjusted to 4 × 1010/ml in PBS. Close contact between bacteria (50 μl) and erythrocytes (50 μl) was achieved by centrifugation at 2,200 × g for 20 min in 96-well U-bottom microtiter plates (Nuncel, delta; Nunc, Roskilde, Denmark). Plates were then incubated at either 37 or 4°C for 3 h without rotation. Pellets were suspended by the addition of 120 μl of cold PBS and were centrifuged again at 2,200 × g and 4°C for 20 min. Supernatants (100 μl) were taken from each well and transferred to another flat-bottom microtiter plate (Nunc-Immunoplate 1; Nunc). Optical density at 545 nm was measured with a multiscan spectrophotometer (Titertek-Multiscan; Flow Laboratories, Inc., McLean, Va.). Thirty-seven strains of EAggEC isolated from different geographic regions showed the CH property when grown in CYE broth supplemented with 1 mM CaCl2 at 37°C for 12 to 22 h with rotation. However, eight strains (518 C3, 1298 C3, 522 C1, 634 C1, H08, W 44-1-3, WC 83-1-2, and PE 464) did not show CH activity under the conditions used here.

For further studies, four strains of EAggEC, PE 7, PE 42, PE 457, and 221, and E. coli 36000 grown in different broth media were treated with erythrocytes from different species. EAggEC showed maximum CH activity with sheep erythrocytes when grown in CYE and nutrient broth, low CH production was observed when EAggEC strains were cultured in Trypticase soy broth, and no CH activity was detected when EAggEC strains were cultured in other media (Fig. 1). Bacterial static culture in CYE or nutrient broth at 37°C did not show any CH activity. Strains of EAggEC grown in CYE broth showed reduced CH activity with rabbit erythrocytes, and very low or no CH activity was detected when the same EAggEC strains were tested with other erythrocytes studied here (Fig. 2). We also tested the hemagglutinating ability of the four CH-positive strains and eight CH-negative strains described above by using 2% erythrocytes as described by Yamamoto et al. (15). For the hemagglutination assay, strains were tested with all of the erythrocytes used here. Eight strains of EAggEC grown in Mueller-Hinton broth showed poor hemagglutination activity, whereas another four strains agglutinated all of the erythrocytes.
Cytes except those from rabbits and showed β-mannose-resistant hemagglutination with guinea pig erythrocytes. The ability to produce CH appeared to be closely dependent on culture conditions and erythrocytes obtained from different species. Bacteria (four strains) cultured in LB broth also showed the hemagglutination property, but CH activities were not detected when the bacteria were grown in Mueller-Hinton or LB broth. EAggEC strains cultured in CYE broth containing CaCl₂ exhibited poor hemagglutination with all of the erythrocytes, but bacteria grown under the same condition showed a high level of CH activity with sheep erythrocytes. These and other studies (9, 15) demonstrate that EAggEC strains exhibit different surface properties under different culture conditions and that the CH receptor also varies among different animal species. However, bacteria grown in CYE broth without CaCl₂ showed reduced or no CH activity with sheep erythrocytes, suggesting that the role of calcium ions is to promote cellular changes in EAggEC, as was demonstrated previously (2). Bacteria showed maximum CH activity at 37°C, and CH activity was absent when tested at 4°C with bacteria cultured at 37°C. However, CH activity could be partially restored by changing the growth temperature to 37°C for 3 h before incubation with erythrocytes. This finding indicates that CH production may be thermoregulated, as has been suggested previously (11). No CH activity was detected when bacterial suspensions were mixed with only sheep erythrocytes and incubated at 37°C for 3 h. So, close contact between bacteria and erythrocytes is important for promoting CH production by EAggEC. To study the effect of pH on CH production, bacteria were grown in CYE broth with 1 mM CaCl₂ at pH 6.5 to 8.5. The optimum pH for the production of CH was 7.2 to 7.6, and CH activity decreased at lower or higher pHs. Treatment (7) of EAggEC with proteinase K (Sigma Chemical Co.) (100 mg/ml at 37°C for 4 h) inhibited CH production, but trypsin (Sigma) (100 mg/ml) and neuraminidase (Sigma) (100 U at 37°C for 4 h) did not inhibit the CH activity. Enzyme treatment of EAggEC did not abolish hemagglutination but did reduce the hemagglutination titer (unpublished data). However, no CH production was observed when bacteria were heated at 85°C for 1 h or 100°C for 10 min. So, the CH molecule may be heat sensitive and a protein in nature. The hemagglutination property of the bacteria was also lost at this temperature, but in our experiment, no correlation between hemagglutination and CH activity was observed. The hemolytic

FIG. 1. CH activity of strains (PE 7, PE 42, PE 457, and 221) of EAggEC grown in brain heart infusion broth (BHI), CYE broth, LB broth, Mueller-Hinton broth (MH), nutrient broth (NB), and Trypticase soy broth (TSB). E. coli 36000 was used as a negative control. Bacteria grown at 37°C for 18 h were treated with sheep erythrocytes, and optical density (OD) at 545 nm was measured. The data are the means ± standard deviations of three replicate experiments.

FIG. 2. CH activity of EAggEC against erythrocytes from different species. E. coli 36000 was used as a negative control in the assay. Bacteria cultured in CYE broth in the presence of 1 mM CaCl₂ were tested with guinea pig ( ), rabbit ( ), rat ( ), sheep ( ), and human type O ( ) erythrocytes. Optical density (OD) at 545 nm was measured. The data are the means of three separate experiments.
activity was observed only when close contact between EAsgEC strains and erythrocytes was obtained. Accordingly, centrifugation-induced interaction between bacteria and erythrocytes allowed demonstration of this contact hemolysis.

EAsgEC strains showed diverse functions, possibly because of the varied expression of surface receptors in different culture conditions. The conserved 60-MDa plasmid of EAsgEC (3) may have some role in the variable functions. Strains of EAsgEC cultured in CYE broth with 1 mM CaCl₂ showed CH production superior to that of those cultured in nutrient broth (Fig. 1). Higher amounts of amino acids present in CYE medium may influence the CH production by these organisms. The role of calcium ions may be important for these EAsgEC strains as the bacteria promote cellular changes in vitro (2). It has been suggested that the 60-MDa plasmid is highly conserved among strains of EAsgEC (3), and aggregative adherence is mediated by this plasmid (9). Our study suggests that CH production by these strains may be plasmid mediated. It has been postulated that the degree or site of expression of virulence factors is variable, and the presence of toxin receptors may be required for the development of EAsgEC-mediated diarrhea (5). It has also been postulated that EAsgEC strains in close proximity to the enterocyte surface may produce some exotoxins (12). In our study, it seems that bacteria, because of the property of aggregative adherence, may serve to create a microenvironment whereby CHs are secreted in close contact to the erythrocytes. In addition to heat-stable and heat-labile toxin produced by EAsgEC strains (2, 12), plasmid-mediated and thermoregulated CH production may be a factor for the pathogenesis of these organisms. Further investigation will show the mechanism of CH production by EAsgEC.

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