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Hemagglutinating Properties of Enteroaggregative *Escherichia coli*

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Many intestinal bacterial pathogens possess hemagglutinating properties, which are indicative of their adhesive properties to the intestinal mucosal surface. To understand the bacteria-mucosa interaction, 41 strains of enteroaggregative *Escherichia coli* (EAggEC), a recently described category of diarrheagenic *E. coli*, isolated mostly from children with diarrhea in Bangladesh, India, Thailand, Central America, and South America were screened for mannose-sensitive hemagglutination and mannose-resistant hemagglutination of erythrocytes from humans, rats, mice, sheep, cattle, and rabbits. Some strains demonstrated mannose-sensitive hemagglutination of erythrocytes. Most isolates showed mannose-resistant hemagglutination of erythrocytes from all species except rabbits. The hemagglutination patterns could be classified into 18 groups. Studies with three selected isolates suggested that hemagglutinins are cell bound and are protein in nature. On the basis of the pattern of inhibition of hemagglutination by various chemicals, 39 isolates were classified into 19 groups. Hemagglutinations of many isolates were inhibited by sialic acid-containing compounds, suggesting that these compounds may be the receptors for these organisms on erythrocytes and possibly on the intestinal mucosa. These data indicate that strains of EAggEC are a heterogeneous group of organisms with different types of hemagglutinins or adhesins for the intestinal mucosal surface. Also, the adhesion characteristics of EAggEC strains may be too complex to be assessed by simple hemagglutination tests.

The four well-recognized classes of *Escherichia coli* that cause diarrhea are enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, and enterohemorrhagic *E. coli* (13). Recently, a fifth category of *E. coli* recognizable by its aggregative or "stacked-brick" type of adherence to cultured mammalian cells has been identified as a causative agent of diarrhea in children in South and Central America (4, 18), India (3), Bangladesh (10), and the United Kingdom (17). However, its role in the causation of diarrhea in children in Thailand is uncertain (5). Because of their characteristic aggregative type of adherence, these *E. coli* strains have been referred to as enteroaggregative *E. coli* (EAggEC) (3).

Adherence to the surface of the intestinal mucosa is an essential step in the pathogenesis of enteric infections. For many intestinal bacterial pathogens, a correlation between hemagglutinating ability and adhesiveness has been shown (2, 8). Since erythrocytes from different species of animals also possess different receptors, the bacteria-erythrocyte interaction gives a clue as to the nature of the receptors for these pathogens in the intestinal mucosa (1, 6, 16). Moreover, different adhesins of the same bacterial species may be identified by studying the pattern of interaction with erythrocytes from different animal species (7, 8). Several studies suggest that EAggEC strains possess hemagglutinating activity (12, 14, 17–19). Two studies with single isolates have shown a close relationship between the production of fimbriae, aggregative adherence (AA), and hemagglutination (HA) (14, 19). In one of those studies, the properties listed above were shown to correlate with adherence to the human intestinal mucosa (19). It is obvious that detailed studies on the hemagglutinating abilities of EAggEC strains will contribute toward understanding the nature of the adhesins of EAggEC and of EAggEC-mucosa interactions. In the present report, we present data on the hemagglutinating properties of a collection of EAggEC strains isolated from various geographical locations.

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MATERIALS AND METHODS

Bacteria. The EAggEC strains originated from various geographical locations and are listed in Table 1. The Bangladeshi strains were obtained from our own previous studies (10); the Mexican strain was obtained from M. Levine, Center for Vaccine Development, University of Maryland, Baltimore; Peruvian strains were obtained from M. Penny, Institute de Investigacion, Nutricional, Lima; Indian strains were obtained from M. K. Bhan, All India Institute of Medical Sciences, New Delhi; and the Thai strains were obtained from P. Echeverria, Armed Forces Research Institute of Medical Sciences, Bangkok. All isolates except those from Thailand originated from children with diarrhea. Among the Thai isolates, those with the prefix W were from children with diarrhea and those with the prefix WC originated from healthy children. All the strains were confirmed as EAggEC by a HEp-2 cell adherence assay (10) and a DNA probe assay (9).

Bacterial cultures. For the HA assay, bacteria were grown on Trypticase soy agar supplemented with 0.6% yeast extract (TSA; GIBCO Laboratories, Grand Island, N.Y.) for 20 h at 37°C or in Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich.) shaker cultures for 20 h at 37°C. The harvested bacteria were washed once in phosphate-buffered saline (PBS; pH 7.2) and were suspended in the same buffer to a density of approximately 10^{10} CFU/ml.

Erythrocytes. Erythrocytes were obtained from various animal species including humans (group O), rabbits, guinea pigs, rats, mice, sheep, and cattle. A 2% (vol/vol) erythrocyte suspension in PBS was used for the assays.

HA assay by slide test. The slide agglutination test was carried out by mixing 10 μl of erythrocytes with an equal volume of bacterial suspension on a slide and observing the slide for agglutination for 1 min. Agglutination was scored on a scale of 0 to 4+ depending on the rapidity of the reaction. Bacteria were examined for mannose-resistant HA (MRHA) by first mixing 10 μl of a 2% solution of D-mannose (Sigma, St. Louis, Mo.) with an equal volume of bacterial suspension on a slide for 5 min; this was followed by the addition of 10 μl of erythrocyte suspension.

Effects of inhibitors. The sensitivity of HA of rat erythrocytes only (because the majority of isolates agglutinated these cells) to various chemicals was tested by a slide test as described above for D-mannose sensitivity. The effects of 2% (wt/vol) solutions of the following chemicals were tested: D-fucose, D-galactose, *N*-acetylneuramin-lactose, *N*-acetylneuraminic acid, bovine mucin, bovine serum albumin, and fetuin (fetal calf serum) (all from Sigma), α_1-glycoprotein (Scottish Blood-transfusion Association, Edinburgh, United Kingdom), and human mucin prepared as described previously (15).

Microtiter HA assay. Samples for HA were titrated in a 96-well U-bottom microtiter plate (Cooke, Alexandria, Va.). Serial doubling dilutions of the bacterial suspension (starting concentration, 10^{10} CFU/ml) in PBS containing 2% D-mannose were made in 25-μl volumes, after which an equal volume of 2% rat erythrocytes was added to each well. The plate was incubated at 4°C for 4 h, and the results were read. The reciprocal of the highest dilution of the bacterial suspension giving complete HA was taken as the titer.

Effects of physicochemical agents on HA. On the basis of the results of initial screening studies, three isolates ($950C_3$, $66C_1$, and $180C_3$) and rat erythrocytes were chosen for further studies. These isolates were chosen because they belonged to the most common HA group. Since HA patterns differed little when grown in MHB or on TSA, the former was chosen (see Results). The HA assay was carried out in the presence of 2% D-mannose. For the sections below describing shaking, heat treatment, enzyme treatment of bacteria, and treatment of bacteria with sodium periodate and EDTA, the bacteria used were grown in MHB with shaking at 37°C for 20 h and were then suspended in PBS at a concentration of 10^{10} CFU/ml. The various chemicals used were dissolved in PBS.

(i) Growth temperature. Bacteria were grown with shaking in MHB at 26, 37, and 42°C for 20 h. HA titers were determined by the microtiter assay.

(ii) Centrifugation. After growth in MHB (with shaking at 37°C for 20 h), bacteria were pelleted at 6,000 × *g* for 10 min at room temperature and the supernatant was tested for HA by the microtiter assay (11).

(iii) Shaking. Five milliliters of bacterial suspension was vigorously shaken at 600 rpm in a gyratory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C for 1 h. The bacteria were pelleted at 6,000 × *g* for 10 min at room temperature, resuspended to the original volume in fresh PBS, and titrated for HA by the microtiter assay. The supernatant obtained by pelleting the bacteria was saved and was also tested for HA by the microtiter assay.

(iv) Heat treatment. Separate tubes of a bacterial suspension were heated in a water bath for 10 min at temperatures ranging from 50 to 100°C at 5°C increments and were then tested for HA by the slide test.

(v) Enzyme treatment of bacteria. Bacteria were treated separately with 2 mg each of trypsin and proteinase K (Sigma) per ml or 100 U of *N*-acetylneuraminidase (Sigma) at 37°C for 4 h. HA was tested by the microtiter assay.

(vi) Treatment of bacteria with sodium periodate and EDTA. Bacteria were incubated separately with 100 mM sodium periodate or 60 mM EDTA at 37°C for 1 h, washed once in PBS, resuspended in PBS to the original concentration, and tested for HA by the slide test.

Performing the tests. All the tests were done in a blinded fashion with the investigator unaware of the identities of the isolates. All the tests were also repeated at least four times to ensure the reproducibility of the results.

RESULTS

The results of mannose-sensitive HA (MSHA) and MRHA with bacteria grown in MHB are given in Table 1. None of the isolates agglutinated rabbit erythrocytes, but erythrocytes from other species were agglutinated. Some isolates produced MSHA of erythrocytes from some species and, at the same time, MRHA of erythrocytes from other species. The majority of isolates showed MRHA of erythrocytes from at least four species, including humans, rats, mice, and sheep. The four Thai isolates from children without diarrhea also possessed HA activity like the diarrheal isolates. Many of the isolates with different serotypes had identical HA patterns. Similar results were obtained for the HA patterns of bacteria grown in MHB or on TSA. Differences were observed only with two isolates: the Bangladeshi isolate $518C_3$ and the Mexican isolate 221. The Bangladeshi isolate showed MRHA of guinea pig erythrocytes when grown on TSA, but was negative when grown in MHB. The Mexican isolate showed MSHA of bovine erythrocytes when grown on TSA, but MRHA when grown in MHB, in addition to the HA properties listed in Table 1.

When the isolates were grouped according to MRHA of erythrocytes, 18 different groups were observed. Seventeen of the 41 isolates studied belonged to group 1 (Table 2). Two strains ($518C_3$ and $522C_1$) did not agglutinate rat erythrocytes. Inhibition of HA of rat erythrocytes for the remaining 39 isolates by various chemicals resulted in 19 patterns (Table 3). Comparison of HA patterns (Table 2) and the inhibition of HA patterns by various chemicals (Table 3) did not show any relationship between the two.

Effects of physicochemical factors on HA. (i) Growth temperature. Three isolates, $950C_3$, $66C_1$, and $180C_3$, produced HA titers of 1:256 when grown at 37°C, but no HA or lower HA titers (1:8 to 1:16) when grown at 26 or 42°C.

(ii) Centrifugation. The supernatant of the growth medium did not possess any HA activity.

(iii) Shaking. Before and after shaking, HA titers remained at 1:256 for the three strains examined. The supernatants were negative for HA.

(iv) Heat treatment. Heating of bacteria up to 80°C did not affect HA, but heating at 85°C and beyond destroyed HA activity.

Enzyme treatment of bacteria. Treatment with trypsin and proteinase K reduced the HA titer from 1:256 to 1:32 to 1:16 for the three bacterial isolates, but treatment with neuraminidase had no effect.

Sodium periodate and EDTA treatment of bacteria. Treat-

TABLE 1. MSHA and MRHA of erythrocytes by EAggEC after growth in MHB

Source and strain	Serotype	MSHA of erythrocytes from[a]:						MRHA of erythrocytes from:					
		GP	R	M	H	S	C	GP	R	M	H	S	C
Bangladesh													
$950C_2$	O126:H27	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$66C_1$	O?:H−	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$518C_3$	O6:H16	0	0	0	−	−	−	0	0	0	4+	4+	4+
$66C_3$	O113:H−	−	−	−	−	4+	4+	4+	4+	4+	4+	−	−
$180C_3$	O5:H11	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$950C_3$	O113:H27	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$130C_2$	O?:H18	−	−	−	−	0	0	4+	4+	4+	4+	0	0
$1429C_2$	O77:H18	−	−	−	−	4+	4+	4+	4+	4+	4+	−	−
$300C_3$	O126:H27	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$1210C_2$	O17,O106:H18	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$186C_1$	O86:H−	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
$779C_2$	O141:H49	4+	4+	4+	4+	−	−	−	−	−	−	4+	4+
$779C_1$	O141:H49	4+	4+	4+	4+	−	−	−	−	−	−	4+	4+
$396C_1$	O126:H27	4+	−	−	−	−	−	−	4+	4+	4+	4+	4+
$1298C_3$	Oru:H7	4+	4+	4+	4+	0	0	−	−	−	−	0	0
$522C_1$	O153:H26	4+	0	0	0	0	0	−	0	0	0	0	0
$634C_1$	O91:H12	0	−	−	0	−	−	0	3+	2+	0	4+	4+
Peru													
75525	O73:H18	0	−	−	−	−	−	0	4+	4+	4+	4+	4+
75527	O?:H13	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
75530	O134:H27	0	−	−	−	−	−	0	4+	4+	4+	4+	4+
75532	O86:H18	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
75535	O?:H27	−	−	−	−	0	−	4+	4+	4+	4+	0	4+
Mexico													
221	O92:H33	4+	4+	4+	4+	4+	−	−	−	−	−	−	4+
India													
F01	O77:H18	−	−	−	−	−	−	2+	4+	4+	4+	2+	4+
F02	O51:H11	4+	4+	4+	−	4+	−	−	−	−	4+	−	4+
F03	O4:H7	4+	−	4+	−	4+	−	−	4+	−	4+	−	4+
F04	O69:H11	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
F05	O9AB:H18	−	−	−	−	−	0	4+	4+	2+	4+	2+	0
F06	O44:H18	−	4+	−	−	−	−	2+	−	4+	4+	2+	4+
H07	O162:H−	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
H08	O111:H−	−	−	−	0	−	0	4+	4+	4+	0	4+	0
Thailand													
W6-1-5	Oru:H2	4+	4+	4+	−	4+	−	−	−	−	4+	−	4+
W44-1-3	O4,O36:H18	0	−	−	−	0	0	0	4+	4+	4+	0	0
W253-1-1	O3:H2	0	−	−	−	−	−	0	3+	3+	4+	4+	4+
W309-1-1	O130:H27	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
W365-1-5	O17-H−	−	−	−	−	−	0	4+	4+	4+	4+	4+	0
WC83-1-2[b]	O15:H18	0	−	−	−	−	0	0	4+	4+	4+	4+	0
WC99-1-5[b]	O38:H9	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
W232-1-3	O127:H21	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
WC310-1-1[b]	O?:H21	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+
WC354-1-3[b]	O86:H19	−	−	−	−	−	−	4+	4+	4+	4+	4+	4+

[a] GP, guinea pig; R, rat; M, mouse; H, human O type; S, sheep; C, cattle. HA with 2% (vol/vol) erythrocytes is graded from a rapid (4+) to a negative (−) reaction. 0, no HA activity.

[b] These isolates were from apparently healthy children; the remaining isolates were from children with diarrhea.

ment with sodium periodate or EDTA did not reduce HA activity.

DISCUSSION

Our results show that most isolates of EAggEC demonstrate MRHA of erythrocytes from various sources including humans, rats, mice, and sheep, but not rabbits. Lack of HA of rabbit erythrocytes by an EAggEC isolate from Thailand has been reported by Yamamoto et al. (19).

The results in general indicated that HA is a strong property of EAggEC, including those four Thai isolates obtained from children without diarrhea. Furthermore, the results are in agreement with those of limited studies with isolates from India (12), South America (18), and the United Kingdom (17). However, Scotland et al. (17), after studying 33 isolates of EAggEC, found that all of them caused MRHA of rat erythrocytes, but agglutination of erythrocytes from guinea pig, bovine, and human species was either variable or absent. They speculated, therefore, that MRHA of rat erythrocytes may be diagnostic of EAggEC. Of 41 isolates tested in the present study, however, only 32 showed MRHA of rat

TABLE 2. Classification of 41 isolates of EAggEC on the basis of MRHA of erythrocytes from various species

Group	MRHA of erythrocytes from[a]:						No. of isolates	Isolates
	GP	R	M	H	S	C		
1	+	+	+	+	+	+	17	$950C_2$, $66C_1$, $180C_3$, $950C_3$, $300C_3$, $1210C_2$, $186C_1$, 75527, 75532, F01, F04, H07, W309-1-1, WC99-1-5, W232-1-3, WC310-1-1, WC354-1-3
2	−	+	+	+	+	+	1	$396C_1$
3	0	+	+	+	+	+	4	$634C_1$, 75525, 75530, W253-1-1
4	+	+	+	+	+	0	2	F05, W365-1-5
5	+	−	+	+	+	+	1	F06
6	+	+	+	+	0	+	1	75535
7	+	+	+	+	0	0	1	$130C_2$
8	0	+	+	+	+	0	1	WC83-1-2
9	+	+	+	0	+	0	1	H08
10	+	+	+	+	−	−	2	$66C_3$, $1429C_2$
11	0	0	0	+	+	+	1	$518C_3$
12	0	+	+	+	0	0	1	W44-1-3
13	−	+	−	+	−	+	1	F03
14	−	−	−	+	−	+	2	W6-1-5, F02
15	−	−	−	−	+	+	2	$779C_2$, $779C_1$
16	−	−	−	−	−	+	1	221
17	−	−	−	−	0	0	1	$1298C_3$
18	−	0	0	0	0	0	1	$522C_1$

[a] GP, guinea pig; R, rat; M, mouse; H, human O type; S, sheep; C, cattle. +, positive for HA; −, positive for MSHA; 0, no HA activity even in the absence of D-mannose.

erythrocytes, with 7 more strains showing MSHA of these cells. This finding indicates that MRHA of rat erythrocytes is not a universal property of EAggEC isolates.

The various patterns of MRHA by EAggEC suggest that these bacteria are a heterogeneous group. Among 46 isolates of EAggEC from India and South America, Knutton et al. (12) demonstrated four distinct types of pili by electron microscopy, one of which was a bundle-forming pilus. Nataro et al. (14) studied a single strain of EAggEC and demonstrated that it produces a bundle-forming pilus, which they designated fimbria I of EAggEC. They demonstrated a close correlation between possession of this fimbria, AA, and MRHA of human erythrocytes. They further noted that of the 36 EAggEC probe-positive strains, 34 showed MRHA of human erythrocytes (94% sensitivity). In our study also, of the 41 probe-positive strains, 34 showed MRHA of human erythrocytes (83% sensitivity). Thus, there seems to be a correlation between MRHA and EAggEC probe positivity. Yamamoto et al. (19), working with a single strain of EAggEC, also demonstrated a close association between adherence to human intestinal mucosae and MRHA of human erythrocytes. This strain also possessed a fimbrial structure. It is known that enterotoxigenic *E. coli* strains, with different colonization factor antigens exhibit different MRHA of erythrocytes from various animal species (7, 8). Whether this is also true of EAggEC is not clear at present.

TABLE 3. Pattern of inhibition of EAggEC-induced HA of rat erythrocytes by various substrates

Group	Inhibition of HA by[a]:						Isolates
	D-Mannose	Mucin	Fetuin	α_1-Glycoprotein	*N*-Acetylneuramin-lactose	*N*-Acetylneuraminic acid	
1	−	+	+	+	−	−	$950C_2$, $950C_3$, F04, W253-1-1, $396C_1$
2	−	+	+	+	+	−	$1210C_2$, 75527
3	−	+	+	+	−	+	W309-1-1
4	−	+	+	−	−	−	$66C_3$, $180C_3$, 75535, H07, WC354-1-3
5	−	−	+	+	−	−	F06, W365-1-5
6	−	+	+	−	+	−	75532, F03
7	−	−	+	+	−	+	F01, W44-1-3
8	−	−	+	+	+	+	$186C_1$
9	−	+	−	+	−	−	WC83-1-2
10	+	+	+	+	−	−	$1298C_3$
11	+	−	−	+	−	−	W6-1-5
12	−	+	−	−	−	−	$66C_1$
13	−	−	+	−	+	−	$1429C_2$
14	−	−	−	+	−	−	WC99-1-5, WC310-1-1, $300C_3$
15	−	−	−	+	−	+	W232-1-3
16	−	−	−	+	+	−	$130C_2$
17	−	−	−	−	−	+	F05
18	+	−	−	−	−	−	221, $779C_1$, $779C_2$, F02
19	−	−	−	−	−	−	$634C_1$, 75525, 75530, H08

[a] +, positive for inhibition; −, negative for inhibition.

There was no relationship between the patterns of HA and the inhibition of HA by various chemicals. This again suggests that multiple hemagglutinins and receptors are involved in HA by EAggEC. The HA of strains was not inhibited by simple sugars, D-fucose, or D-galactose, which is in agreement with a previous report of a study with a single isolate (19). Only a few strains showed MSHA of guinea pig erythrocytes, suggesting that these isolates possess type I fimbriae. However, since these strains were positive for AA in a tissue culture assay in the presence of D-mannose, type I pili are not involved in AA.

HA of several strains was inhibited by substances rich in sialic acids, including α_1-glycoprotein, fetuin, and mucin. Few strains, however, were inhibited by sialic acid derivatives per se, such as *N*-acetylneuramin-lactose and *N*-acetylneuraminic acid. These findings suggest that simple sialic acid derivatives may not be receptors for EAggEC on erythrocytes but that larger molecules containing sialic acids could be. HA was not inhibited by bovine serum albumin, suggesting that the inhibition by the other agents was specific.

Studies with three selected strains showed that expression of HA activity was maximal at 37°C, which agrees with the observation of Yamamoto et al. (19) with a single strain of EAggEC. Furthermore, trypsin and proteinase K treatment and heating of bacteria at higher temperatures (≥85°C) either reduced or abolished HA. Treatment of bacteria with neuraminidase, periodate, and EDTA, however, had no effect on HA. These data suggest that hemagglutinins are proteins. Cell-free supernatants did not have any HA activity, nor did vigorous shaking of bacteria result in any decrease in HA activity. These findings indicate that hemagglutinins are firmly cell bound and are not soluble.

In summary, the results of the present study have shown that the majority of isolates of EAggEC from various geographical locations cause MRHA of a variety of erythrocytes, producing different HA profiles and different patterns of inhibition of HA with various chemicals. Sialic acid-containing compounds inhibited the HA of most isolates. These data indicate that EAggEC strains are a heterogeneous group of organisms and may possess different types of adhesins, and for many of the isolates, sialic acid-containing compounds may be the receptors on erythrocytes and possibly in the gut. The data also suggest that the adhesion characteristics of EAggEC may be too complex to be assessed by simple HA tests. We therefore conclude that the development of a HA scheme may not be useful for studying the adhesins of EAggEC and bacteria-intestinal mucosa interactions.

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