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Hemagglutination of Human Group A Erythrocytes by Enterotoxigenic *Escherichia coli* Isolated from Adults with Diarrhea: Correlation with Colonization Factor

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Enterotoxigenic *Escherichia coli* (ETEC) of several different serotypes isolated from adults with diarrhea and known to possess the colonization factor antigen (CFA) were found to cause mannose-resistant hemagglutination (HA) of human group A erythrocytes. CFA-negative *E. coli* isolated during the same study did not possess the mannose-resistant hemagglutinin, although some non-ETEC, CFA-negative isolates did exhibit mannose-sensitive HA activity. The mannose-resistant hemagglutinin of ETEC was found to possess many characteristics previously associated with CFA, which is a surface-associated fimbriate heat-labile antigen, and the functionally and morphologically similar K88 and K99 antigens of animal-specific ETEC. Mannose-resistant HA and CFA titers were maximal when ETEC cells were grown on an agar medium (CFA agar) composed primarily of 1% Casamino Acids and 0.15% yeast extract, pH 7.4. Neither CFA nor HA were produced at a growth temperature of 18°C; HA was completely inhibited by pretreatment of CFA-positive cells with the anti-CFA serum. The mannose-resistant hemagglutinin was lost spontaneously and simultaneously with CFA when clinical ETEC isolates were passaged on artificial medium in the laboratory, indicating plasmid control of both entities. The mannose-resistant hemagglutinin of ETEC was shown to be thermolabile, i.e., sensitive to heating at 65°C, as was the CFA. Also, there was correlation between possession of CFA, as detected serologically and by demonstration of biological activity (adherence in the infant rabbit small intestine), presence of CFA-type fimbriae, and the ability of various *E. coli* isolates to cause mannose-resistant HA of human group A erythrocytes. These results indicate that the mannose-resistant HA of ETEC is another manifestation of CFA.

Enterotoxigenic *Escherichia coli* (ETEC) were first recognized as causative agents of acute diarrhea in domestic animals. Other investigators have since demonstrated that ETEC are associated with acute diarrhea in man and that ETEC account for a significant proportion of diarrhea cases that were previously undifferentiated with respect to infectious agents (5, 10, 16, 17, 31). This is particularly obvious in the area of travelers' diarrhea, or turista (6, 15, 23, 32). Both ETEC and recently described viral agents account for much of the previously undiagnosed acute diarrhea in infants and young children (2, 8, 17, 22, 29). ETEC isolates from both man and animals produce either a heat-stable or a heat-labile enterotoxin, or both, and are thus very similar with respect to enterotoxigenicity (7, 18, 31). With regard to serotype, or O and H antigenicity, ETEC from man and animals differ significantly in frequency of isolation but are not mutually exclusive; i.e., few serotypes have been isolated from both man and animals (24, 25, 28, 31). The relationship between enterotoxigenicity and serotype in ETEC is difficult to elucidate because enterotoxin production is a plasmid-mediated character in *E. coli* (19, 25, 33). However, ETEC do exhibit host specificity as a natural consequence of the fact that colonization of the small bowel plays an essential role in the pathogenesis of ETEC diarrhea in both man and animals (9, 14, 21, 27, 33). At least three different types of ETEC are currently recognized on the basis of surface-associated, fimbriate, heat-labile antigens, which apparently confer host specificity. The K88 antigen of swine-specific ETEC (21, 28), the K99 antigen of cow and sheep-specific ETEC (27, 33), and the colonization factor antigen (CFA) of human-associated ETEC (9, 30) apparently require different receptors for attachment, or adherence,

to intestinal epithelial cell surfaces. Nevertheless, K88, K99, and CFA antigens share a number of properties. Each is plasmid mediated and hence can be lost spontaneously upon subculture of clinical isolates in the laboratory (9, 26, 27, 33). Each has fimbriate morphology and can be recognized as threadlike surface-associated structures in electron photomicrographs (9, 20, 34; D. G. Evans, D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont, submitted for publication). An interesting and useful property of the K88 and K99 antigens is mannose-resistant hemagglutination (HA) of erythrocytes (1, 21, 34). This property aids in the differentiation of these antigens from the so-called common pili of *E. coli*, which mediates HA that is mannose sensitive (21, 34). The subject of the present report is mannose-resistant HA by *E. coli* possessing CFA.

MATERIALS AND METHODS

Bacterial strains, storage, and culture conditions. *E. coli* strain H-10407 (O78:H11) is a well-characterized isolate of ETEC originating in Bangladesh which we previously described as possessing the fimbriate CFA (9). *E. coli* H-10407-P is the spontaneous derivative of strain H-10407, which no longer possesses CFA. *E. coli* Tx-432-2CH (O78:H12) is an antibiotic-resistant enterotoxigenic isolate, from a prolonged nosocomial outbreak of infantile diarrhea, which produces only heat-stable enterotoxin (30). Strain Tx-119-2 is one of the few isolates of this particular strain found to be lacking CFA. *E. coli* isolates designated by the prefix PB were isolated from the stool of adult volunteers participating in an investigation of diarrhea, turista, in Mexico (6). Many of these isolates were serotyped by Frits and Ida Ørskov of the WHO International Escherichia Centre, Copenhagen, Denmark. Assays for heat-stable and heat-labile enterotoxin activities are described in detail elsewhere (3, 6). Heat-labile enterotoxin was determined by the Y-1 cultured adrenal tumor cell assay of Donta et al. (4) and confirmed by the newly developed passive immune hemolysis technique (8). For long-term storage, cultures were frozen in CYE medium (13) plus 15% glycerol and kept at −65°C. For routine maintenance, stock cultures were kept at room temperature on 2% peptone–0.5% NaCl–2% agar slants.

Improved agar medium for CFA production by *E. coli*. Maximal production of CFA was obtained by growing cultures on CFA agar. This medium was prepared as a modification of CYE medium (13), which was designed for maximal production of enterotoxin by *E. coli*. CFA agar consists of 1% Casamino Acids (Difco) and 0.15% yeast extract (Difco) plus 0.005% $MgSO_4$ and 0.0005% $MnCl_2$ with 2% agar added. The pH of this medium was not adjusted upward but was used at approximately 7.4.

Preparation of CFA and anti-CFA serum. Specific anti-CFA antiserum was prepared using CFA purified from *E. coli* H-10407 as antigen. CFA was purified by shearing the antigen from H-10407 cells followed by differential ultrafiltration, isoelectric precipitation, and ultracentrifugation into sucrose, as described in detail elsewhere (Evans et al., submitted for publication). Rabbits were hyperimmunized with CFA, which was observed by electron microscopy to be a homogeneous preparation of fimbriate material (Evans et al., submitted for publication).

Anti-CFA serum was also prepared by using *E. coli* strain PB-40 formalinized cells as antigen and adsorbing the anti-PB-40 serum exhaustively with heated and then with living PB-40-P cells. This procedure was carried out as previously described (9) for the preparation of anti-CFA serum in which *E. coli* H-10407 was used as antigen and H-10407-P was used to adsorb the serum. The anti-(PB-40)-CFA adsorbed serum agglutinated living but not heat-killed cells of *E. coli* PB-40.

Bacterial agglutination with anti-CFA serum. *E. coli* isolates were tested for CFA by bacterial agglutination with the anti-CFA serum, using the microtiter technique. Bacterial cells were harvested with 0.1 M phosphate-buffered saline (PBS; pH 7.2) from cultures grown on either 2% peptone–0.5% NaCl–2% agar or the CFA agar for 18 h at 37°C. The cell suspensions were adjusted to an optical density of approximately 2.0 at 640 nm with PBS. Serial twofold dilutions of the anti-CFA serum were prepared in U-well microtiter plates (Linbro Scientific Co., Hamden, Conn.) with PBS plus 0.02% bovine serum albumin as diluent. An equal volume (0.025 ml) of cell suspension was then placed in each well, and the plate was sealed and incubated for 60 min at 37°C. Bacterial agglutination was determined visually, using cells plus diluent alone as controls. The agglutination titer was the reciprocal of the highest dilution of anti-CFA serum producing agglutination. Cells that had been heated at either 100°C for 2 h or at 65°C for 1 h also served as controls.

Tests for HA by *E. coli* cells. Blood was drawn from volunteer donors and placed into a tube containing 1.0 ml of 3.8% citric acid, in distilled water, per 9.0 ml of blood. Blood was diluted 1:4 with PBS to test for HA and 1:4 with 1% mannose prepared in PBS to test for mannose-resistant HA. A rapid, semiquantitative test for agglutination was performed by the slide technique, in which 1 drop of blood (0.025 ml) was placed on a glass microscope slide and 1 drop (0.025 ml) of bacterial cell suspension was then added and mixed. Screening of bacterial colonies for mannose-resistant HA was performed by resuspending the bacteria cells in 0.025 ml of group A blood plus mannose, using a sterile toothpick. The degree of HA was recorded as follows. A 4+ reaction was instantaneous and complete HA involving all visible erythrocytes. Slower or less complete HA was graded 3+, 2+, or 1+, usually indicating a mixed CFA^+/CFA^- population of cells.

Serial dilutions of agar-grown cells were prepared and tested as above for quantitating HA. The number of cells in the suspension was determined by the plate-count technique and HA activity was then defined in HA units. One HA unit is defined as the reciprocal of the smallest number of bacterial cells producing HA times a factor of 10^5; for example, if the smallest number of cells that produced HA was 10^6, the sus-

pension contained 0.10 HA unit of bacteria. It should be noted that HA units do not imply more or less hemagglutinating activity per cell, although this is possible, but rather an expression of the relative number of bacteria in the population that are capable of participating in the HA reaction.

HA inhibition by pretreatment of *E. coli* cells with anti-CFA serum was determined as follows. Serial twofold dilutions of the anti-CFA serum were prepared in tubes with PBS plus bovine serum albumin (0.02%), and an equal volume of cell suspension (2 HA units) was added to each tube. After 1 h at 37°C, equal volumes of pretreated bacteria and human group A erythrocytes in mannose were mixed to test for HA. The HA inhibition titer was the reciprocal of the highest dilution of serum that completely blocked the HA reaction.

Assay for adherence of *E. coli* in the infant rabbit model. Intestinal adherence of *E. coli* was determined by the indirect immunofluorescence technique as follows. Three-day-old rabbits were obtained and fasted for 24 h before administration of the bacteria. Two milliliters of a 4-h brain heart infusion broth (Difco) culture of *E. coli*, undiluted, was administered directly into the intestinal lumen as previously described (9; Evans et al., submitted for publication). The animals were sacrificed 3 h postadministration. The small intestine of each was divided into four segments of approximately equal length, and small samples of each segment were rapidly frozen in liquid nitrogen. From these were prepared thin (5- to 8-μm) cross-sections, using an IEC cryostat microtome. Sections were fixed in acetone, air dried, washed in PBS, and exposed first to antibody against the appropriate *E. coli* somatic antigen and then to goat anti-rabbit gamma globulin (globulin fraction; Cappel Laboratories, Inc., Downingtown, Pa.) conjugated with fluorescein isothiocyanate. The goat antiserum was adsorbed with an acetone-dried powder of rabbit muscle to reduce nonspecific fluorescence. Antibody-treated sections were mounted in glycerol-PBS (3:1) and observed by fluorescence microscopy.

RESULTS

Mannose-resistant HA of human erythrocytes by *E. coli* possessing CFA. One of the distinguishing characteristics of the K88 antigen of swine-specific *E. coli* is that these fimbriate structures mediate mannose-resistant HA of guinea pig erythrocytes (21, 34). This has provided a convenient laboratory tool for differentiating K88 fimbriae from the so-called common pili, which are morphologically similar to K88 but mediate mannose-sensitive HA (21). We found that CFA-positive ETEC such as the prototype strain H-10407 exhibited mannose-resistant HA of guinea pig erythrocytes but in an inconsistent fashion, depending, for the most part, on individual animals, and rarely to the extent of K88-positive *E. coli* controls. In the search for a suitable type of blood to perform HA tests for CFA, we found that human erythrocytes sometimes exhibited a strong mannose-resistant HA reaction when mixed with CFA-positive *E. coli*. Subsequently, this variation was found to be dependent on the blood type of the donor. As shown by the representative results listed in Table 1, group A blood cells consistently hemagglutinated with CFA-positive *E. coli* cells. Although not listed in Table 1, it was observed that non-enterotoxigenic clinical isolates, which were negative for CFA, cometimes exhibited mannose-sensitive HA.

In searching for a convenient culture medium for the detection of CFA on *E. coli* isolates, it was found that 2% peptone–0.5% NaCl–2% agar was considerably more effective for CFA detection than either MacConkey or tergitol agar medium (Evans et al., submitted for publication). However, maximum CFA titers have been obtained with an agar medium prepared by incorporating 2% agar into a modification of the CYE liquid medium, which we previously described for maximum yields of enterotoxin (13). Table 2 shows the results obtained with two CFA-positive strains of *E. coli*, H-10407 and PB-40. These data show a direct correlation between the presence of CFA as determined serologically and mannose-resistant HA activity with human group A erythrocytes.

Although mannose-resistant HA can be demonstrated by the simple slide agglutination method, it was sometimes desirable to obtain a more quantitative expression of HA. This was accomplished by expressing HA in units defined as the reciprocal of the smallest number of bacterial cells producing HA times a factor of 10^5.

TABLE 1. *Mannose-resistant agglutination of human erythrocytes by CFA-positive E. coli and comparison of human blood groups A, B, and O*

E. coli strain	Degree of HA[a] with human blood:		
	Type A	Type B	Type O
PB-455	1+	–	–
PB-312	3+	–	3+
PB-379	2+	1+	2+
PB-40	4+	–	2+
PB-66	3+	–	3+
PB-182	3+	1+	3+
PB-60	4+	–	2+
PB-200	4+	1+	2+
PB-418	4+	–	2+
PB-426	4+	1+	1+
TX-432-2CH	4+	1+	2+

[a] All tests were performed with and without mannose with no difference in results. All cultures were grown on 2% peptone–0.5% NaCl–2% agar, and cell suspensions were adjusted to the same density (4+ = maximum HA).

TABLE 2. *Effect of culture medium on the detection of CFA of E. coli and on mannose-resistant HA of human group A erythrocytes*

Culture medium	*E. coli* H-10407		*E. coli* PB-40	
	Agglutination titer with anti-CFA serum	HA units	Agglutination titer with anti-CFA serum	HA units
MacConkey agar	<1:2	0.002	<1:2	0.002
Tergitol agar	1:16	0.008	1:2	0.003
2% Peptone agar	1:128	0.017	1:128	0.017
CFA agar	1:512	0.666	1:512	0.476

This derives from the observation that approximately 10^5 *E. coli* H-10407 cells grown on CFA agar agglutinate human type A erythrocytes. For example, in Table 2 it can be seen that 6.0×10^6 H-10407 cells grown on the peptone-based agar medium produced approximately the same degree of HA as 1.5×10^5 cells grown on the CYE-based agar medium, and therefore the peptone-grown cells demonstrate 0.017 HA unit as compared to 0.666 HA unit of activity with the CYE-grown cells.

For CYE-agar grown cells the following relationship is apparent. Cultures that produced a 4+ reaction by the slide HA technique exhibited a 1:256 or greater titer when used as antigen to titrate the anti-CFA serum and required no more than approximately 10^6 cells to produce the HA reaction (i.e., HA units of 0.100 or greater). Cultures that produced a 2+ or 3+ (moderate to weak) HA reaction by the slide technique exhibited titers of 1:128 or less with anti-CFA serum and require greater than 10^6 cells to produce the HA reaction (i.e., HA units generally less than 0.100). With type A human erythrocytes an HA reaction of less than 4+ by CYE-agar grown cells usually indicates a mixed (CFA^+/CFA^-) culture, as discussed below.

Effect of low incubation temperature on the production of CFA and hemagglutinin by *E. coli*. We recently reported that CFA, like the K88 and K99 antigens of animal-specific ETEC, is not detectable on *E. coli* cells grown at 18°C (1, 27, 28). This provided an opportunity to test for a correlation between production of CFA, using specific antiserum, and production of the mannose-resistant hemagglutinin. Six CFA-positive strains of *E. coli* were grown on the CFA agar at 37 and 18°C, respectively, with the result that none of these strains possessed HA activity or detectable CFA when grown at the lower temperature (Table 3). This is a strong indication that the mannose-resistant hemagglutinin is a function of the surface-associated CFA.

Thermolability of mannose-resistant hemagglutinin of CFA-positive *E. coli*. We reported recently that the CFA of *E. coli* is thermolabile, i.e., destroyed by heating the cells for 1 h at 65°C (Evans et al., submitted for publication). The data in Table 4 show that mannose-resistant HA of CFA-positive *E. coli* is also abolished by heat treatment at 65°C. Various isolates demonstrated the same pattern of thermolability as a function of time. This pattern was similar to that previously observed when specific antiserum was used to detect the CFA.

Spontaneous loss of CFA and mannose-resistant hemagglutinin. Production of colonization factors by ETEC is controlled by specific plasmids, which are spontaneously lost by many clinical isolates upon subculture in the laboratory (19, 25, 26, 33). Although loss of CFA production by *E. coli* is a disadvantage in many respects, such as a major source of variability of CFA assay results, the phenomenon provides a useful tool for investigating biological properties that relate to the possession or loss of CFA. For example, Table 5 lists the results of an investigation testing the idea that if mannose-resistant HA is a function of CFA, both manifestations, reactivity with anti-CFA serum and HA, should be lost simultaneously. The data show that laboratory stock cultures of *E. coli* H-10407 and four other isolates, from Mexico, contained a mixed population of CFA-positive and -negative bacteria, in varying proportions. Note that the CFA-positive isolates from these cultures exhibited both higher titers with the anti-CFA serum and higher units of HA activity. All of the CFA-negative isolates from these cultures failed to exhibit mannose-resistant HA. Thus, the CFA and hemagglutinin were spon-

TABLE 3. *Effect of low incubation temperature on the detection of CFA of E. coli and on HA of human type A erythrocytes*

E. coli strain	Cells grown at 18°C[a]		Cells grown at 37°C	
	Agglutination titer with anti-CFA serum	HA	Agglutination titer with anti-CFA serum	HA
H-10407	<1:2	–	1:512	4+
PB-202	<1:2	–	1:256	4+
PB-143	<1:2	–	1:256	4+
PB-418	<1:2	–	1:512	4+
PB-200	<1:2	–	1:256	4+
PB-60	<1:2	–	1:512	4+

[a] All cultures were grown on CFA agar slants; 18°C cultures were grown for 72 h; 37°C cultures were grown for 24 h (4+ = maximum HA). For titration, cell concentrations were adjusted as described in the text and the footnote to Table 6, although suitable yields of cells were obtained at 18°C with the extended incubation time.

TABLE 4. *Thermolability of mannose-resistant hemagglutinin of CFA-positive E. coli*

E. coli strain	HA units of CFA agar culture	HA of human group A erythrocytes after heating cells at 65°C for (min):						
		0	10	20	30	40	50	60
H-10407	0.666	4+	4+	2+	1+	–	–	–
PB-40	0.500	4+	4+	2+	2+	1+	–	–
PB-200	0.400	4+	4+	2+	1+	–	–	–
PB-379	0.175	3+	3+	2+	1+	–	–	–

TABLE 5. *Simultaneous loss of CFA and mannose-resistant HA by CFA-positive E. coli isolates spontaneously converting to CFA-negative derivatives*

E. coli strain	Agglutination titer with anti-CFA serum	HA units
PB-60 whole culture	1:64	0.015
PB-60 isolate	1:512	0.417
PB-60-P isolates	<1:2	0.000
PB-200 whole culture	1:64	0.001
PB-200 isolate	1:512	0.588
PB-200-P isolates	<1:2	0.000
PB-66 whole culture	1:16	0.001
PB-66 isolate	1:256	0.100
PB-66-P isolates	<1:2	0.000
PB-379 whole culture	1:16	0.010
PB-379 isolate	1:512	1.000
PB-379-P isolates	<1:2	0.000
H-10407 whole culture	1:256	0.625
H-10407 isolate	1:512	1.000
H-10407-P isolates	<1:2	0.000

taneously lost and simultaneously recovered by isolating single clones.

Inhibition of mannose-resistant HA of *E. coli* with anti-CFA serum. It was further hypothesized that if the fimbriate CFA is identical to the mannose-resistant hemagglutinin, anti-CFA antibody should inhibit HA of CFA-positive *E. coli* cells. This was tested with several strains of *E. coli* by pre-exposing the bacterial cells to anti-CFA serum prepared as twofold dilutions before adding human group A erythrocytes and mannose. The anti-CFA serum inhibited, with high titers, the HA activity (Table 6). The correlation between agglutination titers with the anti-CFA serum and the HA inhibition activity of the same serum suggests that the CFA is the mannose-resistant hemagglutinin.

Further evidence for the serotype-independent nature of CFA. We report elsewhere (Evans et al., submitted for publication) that possession of CFA by ETEC is independent of *E. coli* serotype. This was concluded from the observation that isolates of several different serotypes agglutinate with antiserum prepared

TABLE 6. *Inhibition of HA of E. coli by anti-CFA serum*

E. coli strain	Agglutination titer with anti-CFA serum	Titer of HA-inhibition of anti-CFA serum[a]
H-10407	1:1,024	1:512
H-10407-P	<1:2	
PB-40	1:512	1:512
PB-40-P	<1:2	
PB-200	1:1,024	1:1,024
PB-200-P	<1:2	

[a] In all cases HA was performed with human type A erythrocytes in the presence of mannose, using approximately 2.0 HA units of bacteria. Bacteria were mixed with equal volumes of twofold dilutions of anti-CFA serum and incubated for 1 h at 37°C before testing for HA.

with purified *E. coli* H-10407 CFA as antigen. All of the serologically CFA-positive *E. coli* tested possessed fimbriae like strain H-10407 and were shown to possess colonization factor activity in the infant rabbit small intestine (Evans et al., submitted for publication). This study has been carried further by preparing CFA-specific antiserum, using a CFA-positive ETEC strain of serotype O20:H$^-$ as antigen. Animals were immunized with living cells of this strain, PB-40, and the resultant immune serum adsorbed exhaustively with both heat-killed and living cells of strain PB-40-P, a spontaneous CFA-negative derivative of PB-40. The data in Table 7 show that this anti-CFA serum, like that prepared with strains H-10407 and H-10407-P, agglutinates *E. coli* of serotypes O78:H11 and O25:H42 as well as the homologous serotype O20:H$^-$. Also, the nonfimbriate and mannose-resistant HA negative spontaneous derivatives of all of these isolates fail to agglutinate with either CFA-specific serum. This further confirms the observation that CFA is independent of *E. coli* O and H antigenicity.

Correlation between mannose-resistant HA and colonization activity in infant rabbits. Table 8 summarizes the data obtained by assaying various CFA-positive, HA-positive strains and CFA-negative, HA-negative strains of *E. coli* for adherence in the infant rabbit small intestine. This shows that the property of

mannose-resistant HA correlates with the biological activity associated with possession of CFA, i.e., the ability to colonize the infant rabbit small intestine. The relationship between CFA, detected serologically, adherence in the infant rabbit, and the presence or absence of fimbriae on ETEC, mostly the same strains used in the present work, has been described elsewhere (9; Evans et al., submitted for publication).

DISCUSSION

HA can be looked upon as an in vitro model for adhesiveness associated with pili or fimbriae of *E. coli*. With the results reported here, we can now state more specifically that mannose-resistant HA is an in vitro model for adhesiveness of ETEC. Interestingly, K88, K99, and CFA demonstrate specificity as hemagglutinins, apparently reflecting the fact that each of these antigens demonstrates host specificity in adhering to intestinal epithelial cells. *E. coli* possessing the K88 antigen readily attach to intestinal epithelial cells of pigs and exhibit mannose-resistant HA of guinea pig erythrocytes (21, 34). *E. coli* possessing the K99 antigen, which mediates attachment to intestinal epithelial cells of calves, exhibits mannose-resistant HA of sheep erythrocytes (1). As reported here, ETEC isolated from cases of acute diarrhea in man and shown to possess CFA exhibit strong mannose-resistant HA of human group A erythrocytes. These results indicate that mannose-resistant HA by CFA of ETEC is probably more than coincidental and that this phenomenon may eventually lead to an understanding of the biochemical basis of epithelial cell attachment by ETEC with fimbriate antigens. For example, type A erythrocytes from approximately a dozen different individual donors have been used in this and other work in our laboratory with no detectable differences in results. This consistency suggests that surface moieties relating to the ABO typing system are involved in the HA reaction with the H-10407-type CFA fimbriae. However, so-called minor human erythrocyte antigens have not as yet been determined for the donors used in the work reported here, so that the biochemical basis for the apparent specificity of the HA reaction remains speculative. The fact that human erythrocytes are well defined antigenically should aid in determining the biochemistry of the adhesiveness of the H-10407-type CFA fimbriae.

The evidence that the CFA of human-associated ETEC is responsible for mannose-resistant HA of human group A erythrocytes can be summarized as follows. The hemagglutinin described here was found to possess all of the properties previously ascribed to CFA (9; Evans et al., submitted for publication), i.e., a parallel effect with respect to growth medium composition, failure of production at 18°C, thermolability, spontaneous loss by clinical isolates of ETEC, serotype independence, and a correlation with the presence of fimbriae and the biological activity defined as colonization in the infant rabbit model. Although not detailed here, it may also be added that since characterization of the mannose-resistant hemagglutinin numerous ETEC isolates have been screened for this activity, and with *E. coli* isolates from adults with turista

TABLE 7. *Demonstration of serotype-independent bacterial agglutination with anti-CFA serum prepared by the adsorption method from an enterotoxigenic CFA-positive E. coli, serotype O20:H⁻*

E. coli strain	Serotype	Agglutination titer with anti-CFA serum prepared against CFA of *E. coli*:	
		H-10407 (O78:H11)	PB-40 (O20:H⁻)
H-10407	O78:H11	1:512	1:1,024
H-10407-P		<1:2	<1:2
PB-40	O20:H⁻	1:512	1:1,024
PB-40-P		<1:2	<1:2
PB-200	O20:H⁻	1:512	1:1,024
PB-200-P		<1:2	<1:2
PB-143	O25:H?	1:512	1:1,024
PB-143-P		<1:2	<1:2
PB-434	O25:H42	1:512	1:1,024
PB-434-P		<1:2	<1:2

TABLE 8. *Correlation between possession of CFA, mannose-resistant HA, and CFA activity in infant rabbit intestine*

E. coli strain	Agglutination titer with anti-CFA serum	HA activity	Adherence in infant rabbit[a]
PB-312	1:128	3+	+
PB-379	1:128	2+	+
PB-40	1:512	4+	+
PB-200	1:256	4+	+
PB-202	1:256	4+	+
PB-64-P	<1:2	–	–
PB-258-P	<1:2	–	–
Tx-432-2CH	1:512	4+	+
Tx-199-2	<1:2	–	–
H-10407	1:512	4+	+
H-10407-P	<1:2	–	–

[a] Adherence in upper small intestine of the infant rabbit was demonstrated by the indirect immunofluorescence technique (Evans et al., submitted for publication).

diarrhea there has been complete agreement between the hemagglutinin and possession of CFA as detected serologically. Also, mannose-resistant HA is abolished by pretreatment of the isolates with anti-CFA serum. Nevertheless, some of the pitfalls of using mannose-resistant HA as a diagnostic tool should be pointed out. The most obvious problem is loss of CFA by laboratory-passed cultures; as a general rule, several single-colony isolates should be tested if the culture does not represent a fresh isolate. Also, optimal HA can be obtained by subculturing on the CFA agar described in this report, although activity is also detectable when the cultures are grown on 2% peptone–0.5% NaCl–2% agar. Ordinary agar media such as MacConkey or tergitol cannot be used for this purpose.

That CFA is a mannose-resistant hemagglutinin is indeed fortuitous, since this provides a simple technique for the rapid screening of numerous *E. coli* isolates for CFA and, most importantly, a technique that is both economical and immediately available. We are currently investigating various approaches to detecting and quantitating anti-CFA antibody in human sera with the expectation that information about the humoral response of CFA will be very useful in further defining the role of CFA in virulence of ETEC in man. There are yet many questions to be answered before enteropathogenicity can be fully defined with respect to enterotoxigenicity, intestinal colonizing activity, and virulence in *E. coli*. Our experience indicates that this will be particularly complicated in the case of the more susceptible, or less resistant, infant or young child, since we must deal with the question of colonization by *E. coli* that do not produce enterotoxin. There is also the possibility that some strains of CFA-positive *E. coli* may produce enterotoxin activities other than those currently identifiable. Also, it may be expected that intestinal colonization alone would be more likely to cause overt illness in an infant than in a mature, healthy adult. Assuming changes in the nature of the intestinal substrate with age, one might predict the existence of CFA-type virulence factors that confer enteropathogenicity for infants but not for adults. Thus we continue to explore the possibility that non-H-10407-type colonization factors may exist on clinical *E. coli* isolates from infants and young children.

Another area that deserves further investigation is the genetics of CFA production in *E. coli*, particularly in relation to *E. coli* serotype and enterotoxigenicity. With ETEC isolated from adults with diarrhea we apparently have strains of *E. coli* in which *ent* and CFA plasmids cohabit. This phenomenon in itself raises many interesting questions, particularly in view of the observation that both enterotoxin and CFA production are unstable characteristics in the majority of clinical isolates. We report elsewhere (manuscript in preparation) on the instability of CFA production in ETEC isolates from adults in relation to patterns of loss of enterotoxigenicity and serotype, which we recently described (12).

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