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Infant Mouse Model of Adherence and Colonization of Intestinal Tissues by Enterotoxigenic Strains of *Escherichia coli* Isolated from Humans

J. GOLDHAR, A. ZILBERBERG, AND I. OFEK*

Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

Received 28 August 1985/Accepted 30 December 1985

The ability of enterotoxigenic *Escherichia coli* H10407, which possesses colonization factor antigen I, to colonize the intestinal mucosa of infant mice was considerably better than that of its colonization factor antigen I-negative derivative H10407-P. The latter strain previously was shown to lack cell adhering ability in vitro and to have a diminished capacity to infect human volunteers as compared with the parent strain. D-Mannose blocked both colonization by an enterotoxigenic *E. coli* isolate (801) possessing both mannose-resistant and mannose-sensitive adhesins and the in vitro adherence of the strain to intestinal segments of infant mice. A derivative of another enterotoxigenic *E. coli* strain (lacking both mannose-sensitive and mannose-resistant adhesins obtained by in vivo passage showed a significant increase in colonizing ability in comparison with the parent strain. We conclude that the infant mouse model of infection of intestinal mucosa complemented by in vitro adherence assays with excised intestinal tissue is suitable for the study of the bacterial properties responsible for the various stages of intestinal colonization by human enterotoxigenic *E. coli*.

Adherence is probably an essential and prerequisite step for the colonization of mucosal surfaces by microbial pathogens. For a better understanding of the role of virulence factors involved in the initial steps of the infectious process, in vitro assays for testing bacterial adherence to mucosal cells should be complemented by studies of the colonization of the appropriate mucosal surfaces in animal models (10, 13). Various animal models, including infant mice, have been used to study the role of adherence and colonization in the pathogenesis of enteric infections (3, 7, 8). Recently, we developed in vitro assays to measure the ability of human enterotoxigenic *Escherichia coli* to adhere to the intestinal mucosa of infant mice (16, 17). The present study was undertaken to determine whether infant mice may serve as a model for the colonization of intestinal mucosa by human enterotoxigenic *E. coli* expressing mannose-sensitive and mannose-resistant adhesins. We present data to show the influence of the presence of colonization factor antigen I (CFA/I) on the ability of *E. coli* H10407 to adhere to and colonize mouse intestinal mucosa; our results are in agreement with those obtained by others with infant rabbits and human volunteers (6, 12). Our results suggest that feeding infant mice with various human *E. coli* isolates results in intestinal colonization that can be monitored to distinguish between the initial steps (i.e., bacterial adherence) and the subsequent stages of the colonization process. Furthermore, the model allows for the isolation of isogenic mutants or variants with enhanced colonizing ability for further studies of the factors involved in adherence to and colonization of intestinal mucosa.

MATERIALS AND METHODS

Bacteria. *E. coli* H10407, which possesses CFA/I, and *E. coli* H10407-P, which lacks CFA/I, were obtained from D. J. Evans, Jr., D. G. Evans, University of Texas, Houston. *E. coli* 801 (serotype O4:H40), which is fimbriated, produces heat-stable enterotoxin, and possesses both mannose-sensitive and mannose-resistant hemagglutinins, and *E. coli* 667 (serotype O64:H$^-$), which produces heat-labile enterotoxin, is nonfimbriated, and lacks any known hemagglutinins, were isolated in the Tel-Aviv area (15). Both strains 667 and 801 were chloramphenicol resistant. The bacteria were grown at 37°C on CFA agar (5) and harvested after 24 h of incubation to prepare suspensions in phosphate-buffered saline (0.005 M phosphate, 0.15 M NaCl [pH 7.2]).

Inoculation of infant mice in vivo. The modified method of Baselski and Parker (2) as described previously (17) was used. Briefly, 1 or 2 days after delivery of ICR mice, each family (mother and her infants) was caged separately and fed a normal diet, with the exception that the drinking water of the mice to be used in experiments with *E. coli* 667 and 801 contained 0.2 mg of chloramphenicol per ml.

After 48 h, the litters were separated from their mothers; 3 h later, the infant mice were inoculated orally with 10 μl of a bacterial suspension containing various amounts of bacteria (5×10^1 to 5×10^5) in a 5% solution of either sucrose or D-mannose in water supplemented with 0.001% Evans blue. For testing each inoculum size, 10 mice were used. The inoculated infant mice were returned to their mothers and sacrificed after 3, 24, and 72 h. The bowel segments were removed and opened longitudinally, and two segments (3 cm each) were placed into 2 ml of a 5% saponin solution, incubated for 10 min at room temperature, and agitated on a vortex shaker for an additional 1 min to release the adherent bacteria. The segments dissected 3 h after inoculation were washed in cold saline prior to treatment with saponin. The number of CFU in the saponin wash of each intestine was determined by plating triplicates of MacConkey agar containing 60 μg of chloramphenicol per ml (for strains 667 and 801) and, in parallel, MacConkey agar without the antibiotic.

In all cases, there was no difference between the number of CFU of bacteria grown on medium with or without the antibiotic, suggesting that the infant mice were free of natural colonization by *E. coli*.

To ascertain that the recovered bacteria were of the same original inoculum strain, a number of representative bacte-

* Corresponding author.

TABLE 1. Infectivity in mice of *E. coli* H10407 (CFA/I) and H10407-P

Inoculating dose/mouse	No. of mice infected at indicated time after inoculation with indicated strain[a]					
	3 h[b]		24 h[b]		72 h[b]	
	H10407	H10407-P	H10407	H10407-P	H10407	H10407-P
5×10^1	0	0	0	0	6	0
5×10^2	3	0	5	0	10	2
5×10^3	6	1	8	2	10	3
5×10^4	10	4	10	4	10	7
ID_{50}[c]	2×10^3	1.5×10^5	7.6×10^2	7.2×10^4	<50	1.1×10^4

[a] Ten mice were tested per group.
[b] $P < 0.001$ as calculated by the method of Mantel-Haenszel for a multiple two-by-two table, corrected for continuity, as described by Rimm et al. (11).
[c] Calculated by the method of Reed and Muench as described by Cruikshank et al. (4).

rial colonies were tested routinely for antibiotic susceptibility patterns and serologically identified by slide agglutintion with an appropriate antibacterial serum.

The colonization of intestinal mucosa was assessed after challenge with various doses of bacteria; the results were determined as follows. The number of infected mice was determined for each inoculum size tested; an infected mouse was considered any mouse which showed positive cultures of the inoculated bacteria in the saponin wash of its intestinal mucosa. The 50% infective dose (ID_{50}) was calculated by the method of Reed and Muench as described by Cruickshank et al. (4). The mean number of CFU of the infecting strain per mouse recovered from intestinal mucosa (± standard error) was determined, and the data were statistically evaluated by using the Student *t* test.

RESULTS

Infectivity of *E. coli* H10407 and its derivative H-10407-P. The ID_{50} of strain H10407-P was significantly higher than that of strain H10407 throughout the 3 days after inoculation (Table 1). The mean number of CFU per mouse recovered from the intestinal mucosa 24 and 72 h after inoculation was significantly lower for strain H10407-P than for strain H10407 (Fig. 1). The data indicate that the intestinal colonization of infant mice was significantly lower with mutant strain H10407-P than with parent strain H10407.

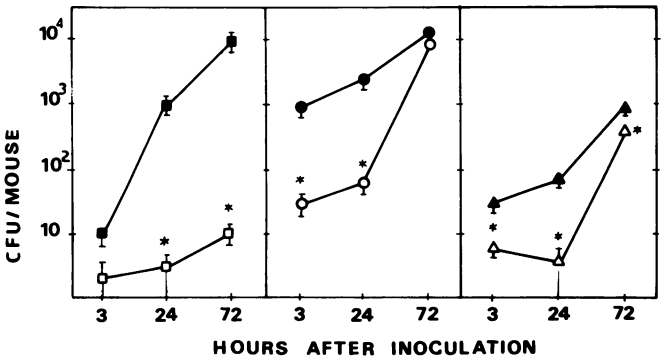

FIG. 1. Number of organisms (mean CFU ± standard error) recovered from the intestines of infant mice 3, 24, and 72 h after inoculation with various *E. coli* strains. Symbols: ■, H10407; □, H10407-P; ●, 801 + sucrose; ○, 801 + D-mannose; △, 667; ▲, 667-3. The mean CFU ± standard error recovered were derived from data obtained from each of 10 mice inoculated with 5×10^3 bacteria at each time. Asterisks indicate *P* values of <0.05.

Influence of the presence of D-mannose in the inoculum on the infectivity of *E. coli* 801. The ID_{50} of strain 801 3 and 24 h after inoculation of mice was significantly higher in the presence of D-mannose than in the presence of sucrose (Table 2). The mean number of CFU per mouse recovered from the intestinal mucosa throughout the 3 days after inoculation of mice with *E. coli* 801 was significantly lower in the presence of D-mannose than in the presence of sucrose (Fig. 1). The data show that D-mannose markedly inhibited the colonization of the mouse intestinal mucosa, mostly during the first 3 h after inoculation. This effect decreased after 24 h.

Selection of the *E. coli* 667 derivative with enhanced colonizing ability. Twenty infant mice were fed with bacterial suspensions containing 50 bacteria per mouse. After 72 h, bacteria were recovered from only one mouse, and after transfer to CFA agar, the organisms designated 667-1 were used to inoculate eight mice with bacterial suspensions containing 5×10^3 bacteria per mouse. The mice were sacrificed after 3 h, and the bacteria recovered in the saponin wash of the intestine were cultivated and used to inoculate 10 mice (6.4×10^2 bacteria per mouse). After 72 h, the bacteria recovered from the intestinal mucosa were designated 667-3.

Bacteria of the first passage (667-1) and of the third passage (667-3) were tested for biotype, spectrum of resistance to antibiotics, agglutination with a specific group antiserum, and hemagglutination of guinea pig, human, and bovine erythrocytes. None of the tested properties of the derivative strains were different from those of parent strain 667.

There was no significant difference in the ID_{50} values or in the mean number of CFU per mouse between derivative strains 667-1 and 667-3 (data not shown).

The ID_{50} of parent strain 667 was significantly higher throughout the 3 days after inoculation of mice than that of derivative strain 667-3 (Table 3). The mean number of CFU per mouse recovered from the intestinal mucosa 24 and 72 h after inoculation was significantly lower for parent strain 667 than for derivative strain 667-3 (Fig. 1). The mean CFU per mouse recovered from mouse intestinal mucosa 3 h after inoculation as a function of the inoculating dose also revealed a marked improvement of the recovery of derivative

TABLE 2. Infectivity in mice of *E. coli* 801 in the presence and absence of D-mannose in the inoculum

Inoculating dose/mouse	No. of infected mice at indicated time after inoculation with strain 801 and indicated supplement[a]					
	3 h[b]		24 h[c]		72 h	
	Sucrose	D-Mannose	Sucrose	D-Mannose	Sucrose	D-Mannose
5×10^1	2	0	4	0	10	10
5×10^2	4	0	7	3	10	10
5×10^3	8	3	8	6	10	10
5×10^4	10	6	10	10	10	10
ID_{50}[d]	7×10^2	2.7×10^4	2.0×10^2	2.0×10^3	<50	<50

[a] Ten mice were tested per group.
[b] $P < 0.001$ as calculated as decribed in Table 1, footnote *b*.
[c] $P < 0.01$ as calculated as described in Table 1, footnote *b*.
[d] Calculated as described in Table 1, footnote *c*.

strain 667-3, as compared with that of parent strain 667 (Fig. 2).

DISCUSSION

There is little doubt that adherence is a prerequisite for the colonization of mucosal surfaces (10, 13). Colonization, however, is a process which includes adherence to tissues and physiological adaptation, leading to the proliferation of bacteria at a particular site (8). The animal model described in this article seems to be suitable for studying these colonization factors of human enteropathogens. We used two approaches to assess the usefulness of the infant mouse model. First, we compared the colonizing capacity of *E. coli* H10407 and its derivative H10407-P. Previous studies have shown that the H10407-P variant, which lacks the CFA/I adhesin that binds the organisms to epithelial cells, failed to produce diarrhea and was cleared from the gastrointestinal tract of infected volunteers considerably more rapidly than was the H10407 parent (12). As for the human volunteers, the H10407-P variant had reduced colonizing ability in mice, reflected both in the mean number of bacteria retained on the intestinal mucosa and in the ID_{50} during the 3 days after infection of the infant mice. In the second approach, we used a specific inhibitor of bacterial adherence to epithelial cells. Previous studies have shown that colonization of the urinary tract can be blocked by injecting *E. coli* suspended in a solution containing analogs of epithelial cell receptors responsible for the adherence of the organisms to the tissues. This was shown for *E. coli* expressing the mannose-sensitive adhesin in mice (1) and for *E. coli* expressing globoside-specific adhesins in mice (14) and primates (9) by using D-mannose derivatives and Gal-Gal disaccharide as inhibitors of epithelial cell adherence, respectively. Similary, D-mannose reduced the intestinal colonization of infant mice infected with *E. coli* 801, which expresses both mannose-sensitive and mannose-resistant adhesins. The effect of the sugar was noticeable mostly during the first 3 h after infection. This observation confirms our previous finding that the adherence of strain 801 to intestinal segments of infant mice was inhibited by D-mannose (16). Thus, the retention of bacteria on the intestinal mucosa 3 h after infection may reflect the adhering ability of the organisms in vivo, and the subsequent persistence in the gut reflects the adherence and physiological adaptation of the organisms to the in vivo environment. This notion is supported by results obtained in our previous study, in which differences in adherence to intestinal segments of infant mice between agar- and broth-grown *E. coli* were reflected in the quantitative recovery of the organisms from the intestines 3 h after inoculation but not 24 or 72 h after inoculation (17).

TABLE 3. Infectivity in mice of *E. coli* 667 and its derivative 667-3

Inoculating dose/mouse	No. of infected mice at indicated time after inoculation with indicated strain[a]					
	3 h[b]		24 h[c]		72 h[c]	
	667	667-3	667	667-3	667	667-3
5×10^1	0	0	0	0	1	10
5×10^2	0	2	0	3	9	10
5×10^3	4	5	1	5	10	10
5×10^4	7	10	4	10	10	10
ID_{50}[d]	1.3×10^4	5.0×10^3	5.0×10^4	2.4×10^3	1.6×10^2	<50

[a] Ten mice were tested per group.
[b] $P < 0.05$ as calculated as described in Table 1, footnote *b*.
[c] $P < 0.001$ as calculated as described in Table 1, footnote *b*.
[d] Calculated as described in Table 1, footnote *c*.

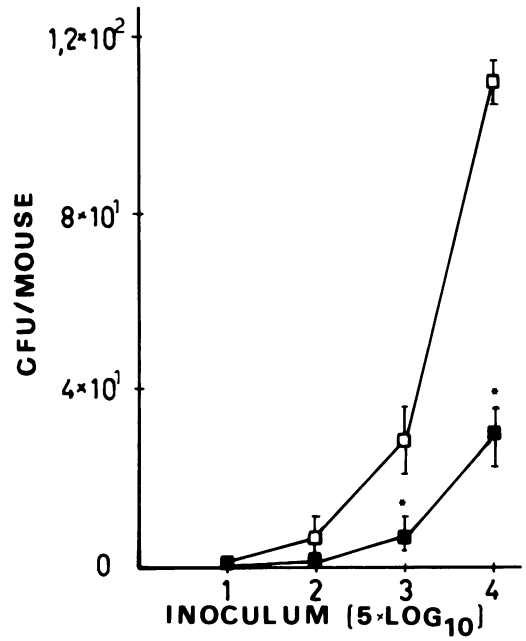

FIG. 2. Number of organisms (mean CFU ± standard error) recovered from the intestines of infant mice 3 h after inoculation with various doses of *E. coli* 667 (□) and its derivative 667-3 (■). The mean CFU ± standard error recovered were derived from data obtained from each of 10 mice for each dose. Asterisks indicate *P* values of <0.05.

The above results encouraged us to use the infant mouse model to select variants possessing enhanced colonizing ability. We used a human isolate of enterotoxigenic *E. coli* 667, which lacks any detectable adhesin(s), as determined by hemagglutination assays, and has a relatively poor colonizing ability in the mouse model (16, 17). The 667-3 derivative obtained after 3 passages in the mouse intestine gained an increased colonizing ability, although it did not exhibit hemagglutination with any of the erythrocytes tested. Careful comparison of the parent and derivative strains by both in vitro and in vivo assays should clarify which factors are responsible for the enhanced colonization as well as at what stage of the colonization process these factors exert their effect.

In summary, it appears that the infant mouse model described in this study, together with the in vitro assay of adherence to mouse intestinal segments described previously (16), is suitable for the study of bacterial properties responsible for the various stages of intestinal colonization by human enterotoxigenic *E. coli*. In particular, it allows the screening potential inhibitors of adherence to intestinal segments in vitro and subsequently the testing of the ability of selected inhibitors to prevent intestinal colonization by bacterial pathogens.

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