Adherence of non-fimbriate entero-invasive *Escherichia coli* O124 to guinea pig intestinal tract *in vitro* and *in vivo*

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Summary. The adherence properties of non-fimbriate entero-invasive *Escherichia coli* O124 in the guinea pig intestinal tract were studied. Quantitative in-vitro determinations were done by incubating radiolabelled bacteria with suspensions of viable intestinal cells released by treating loops of the guinea pig intestine with solutions containing EDTA, dithiothreitol and citrate. Non-bound bacteria were separated from the intestinal cells on a Percoll gradient. Only cells released from the colon, especially from its transverse and descending regions, avidly adhered to *E. coli* O124 (68–79 bacteria/cell), whereas the attachment to ileal cells was negligible. The adherence process was Ca^{++} and temperature-dependent, had an optimal pH of 6.2 and was inhibited by fucose, glucose or mannose. Several pretreatment studies of the bacteria or the colonic cells showed that the adherence was mediated by a carbohydrate-binding protein (adhesin or lectin) on the colonic cells and not on the bacterial surface. Results of studies of in-vitro adherence to intestinal loops and to intact intestinal surfaces correlated well with the in-vitro findings. These results indicate that the adherence of entero-invasive *E. coli* O124 to the gut is similar to the attachment of *Shigella flexneri* and is quite different from that of enterotoxigenic *E. coli*.

Introduction

Escherichia coli is a major component of the normal flora of the intestinal tract (Mackowiak, 1982), but is also an important cause of diarrhoeal diseases (Drachman, 1974). The enteric diseases caused by *E. coli* can be classified into at least two types. Some *E. coli* strains (enterotoxigenic *E. coli*, ETEC) colonise the small intestine, elaborate an enterotoxin and produce a watery diarrhoea in infants and adults; this type is especially common in developing countries and in travellers from developed countries (Guerrant *et al.*, 1975; Sack, 1980). The ability to secrete enterotoxin (either heat-stable or cholera-like heat-labile) is usually plasmid-mediated (Back *et al.*, 1980). Other *E. coli* strains are entero-invasive and cause a dysentery-like syndrome with bloody stool, tenesmus and fever (Guerrant *et al.*, 1975; Cantey *et al.*, 1977).

The adherence of bacteria to epithelial surfaces has been found to be an essential requirement for colonisation (Hartley *et al.*, 1979) and virulence (Cheney *et al.*, 1980; Boedeker, 1982). Attachment to the intestinal epithelium enables the micro-

organisms to resist peristaltic clearing mechanisms and colonise the intestine, sometimes with subsequent enterotoxin production or tissue invasion.

The adherence properties of ETEC have been extensively investigated and depend on bacterial surface appendages such as fimbriae. Several surface antigens (K88 and K99) have been isolated from animal ETEC strains (Isaacson, 1977; Mooi and deGraaf, 1979) and from human strains, in which they are called colonisation-factor antigens (Evans and Evans, 1978; Deneke *et al.*, 1979). Some of them have been purified and well characterised (Isaacson, 1977; Evans *et al.*, 1979). Adherence of *E. coli* to the mucosa of the small intestine without evidence of enterotoxin production or invasion has been described as associated with protracted diarrhoea in infants (Ulshen and Rollo, 1980; Rothbaum *et al.*, 1982). On the other hand, the adherence properties of entero-invasive strains of *E. coli*, whose virulence is not clearly plasmid-mediated (Nandadasa *et al.*, 1981; Silva *et al.*, 1982), has not yet been clarified.

A system for studying the adherence of bacteria to the guinea pig intestinal tract has been described recently (Izhar *et al.*, 1982; Ashkenazi and Mirel-

man, 1984). In the present study the adherent properties of a non-fimbriate entero-invasive *E. coli* strain were examined and compared with those of a non-invasive strain of *E. coli* and a strain of *Shigella flexneri*.

Materials and methods

Adherence of *E. coli* to guinea pig intestinal tract was quantitatively determined with radiolabelled bacteria as follows.

Bacteria

A clinical isolate of *E. coli* O124 was obtained from Dr G. Altman, Department of Microbiology, Tel Hashomer Hospital, Israel, and of *E. coli* O128:H4 was obtained from Dr N. Gerber, Bar Ilan University, Israel. *E. coli* strains were grown in peptone (Difco) 1%, yeast extract 0.5% and NaCl 0.5%. *S. flexneri* was grown in Luria broth, supplemented with 5 mM CaCl₂ and glucose 0.2%. The invasiveness of *E. coli* O124 was repeatedly tested by the guinea pig eye inoculation technique (Sereny, 1957).

Preparation of radiolabelled bacteria

Sixteen hours before each experiment, bacteria were transferred by a sterile wire loop from stock agar slants to freshly prepared media, with the addition of 0.2 ml D-(¹⁴C)-glucose (239 mCi/mmol, 0.1 uCi/ml; The Radiochemical Centre, Amersham), and incubated at 37°C. The bacteria were harvested by centrifugation at 900 g for 10 min and washed three times by suspension in saline and re-centrifugation. The sedimented bacteria were resuspended in isotonic saline and their concentration adjusted photometrically to give a suspension of 2×10^9 bacteria/ml. The radioactivity of the labelled bacteria was determined by counts in a Tricarb liquid scintillation spectrometer (model 3255, Packard Instrument Co., USA) with 10 ml of Triton 100-Toluene scintillation fluid. The specific radioactivity obtained was $c. 10^6$ counts/min/ 10^9 bacteria. Precipitation in 5% trichloroacetic acid showed that >90% of the radiolabelled precursor was incorporated into macromolecular components of the bacteria.

Preparation of intestinal epithelial cells

A suspension of intestinal cells was obtained as described previously (Izhar *et al.*, 1982; Ashkenazi and Mirelman, 1984). The technique of Weiser (1973) was used and sections of the intestine were treated with solutions containing EDTA, citrate buffer and dithiothreitol. The viability of the intestinal cells was monitored by trypan blue exclusion.

Assay for bacterial adherence to epithelial cells

A suspension of epithelial cells (200 µl; 5×10^6 cells/ml)

obtained from different regions of the intestinal tract was incubated with radiolabelled bacteria (100 µl; 2×10^9 bacteria/ml; 200 bacteria/epithelial cell) at 37°C in a rotating rack at 16 rpm. After 45 min the interaction was terminated by adding 2.5 ml of saline and centrifuging at 500 g for 5 min to sediment the epithelial cells; the supernate, containing mainly the nonadherent bacteria, was discarded. The separation of the epithelial cells from the remaining nonadherent bacteria was performed by discontinuous density-gradient centrifugation with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) (Izhar *et al.*, 1982; Ashkenazi and Mirelman, 1984). The layer of epithelial cells with the adherent bacteria was collected and counted for radioactivity.

Assay for bacterial adherence to intact intestinal surface

Sections of guinea pig intestine were obtained after washing the saline. They were opened by a longitudinal incision and placed on a flat plastic surface with the mucosa facing upwards. A cover containing three oval holes with a surface area of 0.38 cm² was screwed on to the mucosal surface. The exposed portion of the mucosa was incubated with radiolabelled bacteria (100 µl; 2×10^9 bacteria/ml) (Izhar *et al.*, 1982). The preparation was incubated at 37°C for 45 min in a humid chamber and the reaction was then terminated by washing the tissue three times with saline. The tissue was taken out of the instrument, the area of intestinal mucosa that had been exposed to the labelled bacteria was cut out and heated at 100°C for 15 min in a tube containing 1 ml of 2% sodium dodecyl sulphate. The adherent radiolabelled bacteria were solubilised under these conditions (Braun, 1975) and their released radioactivity was determined as described previously.

Assay for in-vitro bacterial adherence to intestinal loops

Each guinea pig was anaesthetised and the peritoneal cavity opened. Loops (2 cm long) were created in the intestine by surgical sutures. A suspension of radiolabelled bacteria (200 µl, 10^9 bacteria/ml) was carefully introduced into each loop with a 26-gauge needle; the loops were then replaced in the peritoneal cavity and the abdomen covered for 45 min. Thereafter, each loop was cut from the intestine, washed with saline and then the opened intestine was spread and scraped gently with a glass slide. The tissue was suspended in 5 ml of saline, centrifuged at 500 g for 5 min and the non-adherent bacteria were separated from the intestinal mucosa on the Percoll gradient, as described above. The radioactivity of the radiolabelled bacteria that adhered to the mucosa was determined. A tenth part of the suspension of mucosal scrapings was taken for protein determination (Lowry *et al.*, 1951) and the results were expressed as adherence per g of protein.

Statistical methods

All the experiments were done in triplicate. Results are reported mean $\pm$ standard error of the mean ($M \pm SEM$).

Scanning electron microscopy

After the assay for bacterial adherence, the intestinal cells or intestinal mucosa were washed in saline, fixed with glutaraldehyde 2% for 1 h, and washed with 0.1 M sodium cacodylate buffer at pH 7.2. They were then incubated with osmium tetroxide 1% for 30 min and dried to a critical point. The tissue was fixed to planchets, coated with gold and examined with a JSM-35c scanning electronmicroscope (Jeol, Japan).

Results

The invasiveness of *E. coli* O124 was repeatedly confirmed by producing keratoconjunctivitis in guinea pigs. Electronmicrographs of negatively stained cells revealed that the bacteria did not have any cell surface appendages, and they did not agglutinate mannan-containing yeast cells (Mirelman *et al.*, 1980) or erythrocytes. *E. coli* O124:H4, whose adherent properties were compared with those of *E. coli* O124, was not invasive, possessed type-I fimbriae (Gaastra and deGraaf, 1982) and agglutinated yeast cells and guinea pig, but not human, erythrocytes.

Adherence to intestinal cells

Quantitative examination of the adherence of the bacteria to intestinal cells from various regions of the intestine (table I) showed that *E. coli* O124 adhered well to colonic cells, mainly those released from its transverse and descending regions (68–79 bacteria/cell). Its adherence to ilial cells was negligible (<2 bacteria/cell). *E. coli* O128 showed less

Table I. Adherence of *E. coli* O124 and *E. coli* O128 to guinea pig intestinal cells released from various regions of the intestine*

Region of the intestine	Number of adherent bacteria/cell in tests with	
	<i>E. coli</i> O124	<i>E. coli</i> O128
Small intestine	2 ± 1	19 ± 3
Ascending colon	18 ± 6	14 ± 3
Transverse and descending colon	73 ± 9	42 ± 6

* Radiolabelled bacteria (2×10^8) were incubated with the intestinal cells (10^6) at 37°C for 45 min.

affinity for colonic cells, but adhered well to cells released from the small intestine (17–20 bacteria/cell).

The adherence of *E. coli* O124 to colonic cells was temperature dependent, required Ca^{++} ions (fig. 1), and the optimal pH was 6.2. Significant inhibition of the attachment (*c.* 80%) was achieved by 50 mM fucose and 100 mM glucose (table II). Mannose (100 mM) caused only *c.* 50% inhibition and other

Figs 1a and 1b. The effect of temperature of incubation (fig. 1a) and Ca^{++} concentration (fig. 1b) on the adherence of *E. coli* O124 to colonic cells. Radiolabelled bacteria (200 bacteria/cell) were incubated for 45 min with viable epithelial cells.

carbohydrates had no inhibitory effect. Lipopolysaccharide (LPS) of *E. coli* O124, prepared by the phenol-water extraction method (Westphal and Jann, 1965), was also a potent inhibitor of the adherence: 79% inhibition was achieved at an LPS concentration of 1 mg/ml (table II).

The effect of several pretreatments on the adherence process was examined. Pretreatment of the colonic cells by glutaraldehyde fixation, proteolytic digestion or heat inactivation (Izhar *et al.*, 1982) completely abolished their adhesiveness for the test bacteria. Preincubation of the colonic cells with fucose 10 mg/ml at 37°C for 45 min, followed by washing, also inhibited >90% of the attachment. On the other hand, similar pretreatments of the bacteria did not affect their subsequent adherence to the colonic cells.

Preincubation of the colonic cells with 2×10^8 unlabelled *E. coli* O128 for 45 min competitively inhibited (41%) the subsequent adherence of radiolabelled *E. coli* O124 (fig. 2). *E. coli* O124 also inhibited the adherence of radiolabelled *E. coli* O128 in a converse experiment, indicating that the attachment of these bacteria to colonic cells is probably by the same mechanism.

The adherence of *E. coli* O128 to ileal cells had different characteristics. Mannose was the only carbohydrate which caused >50% inhibition of the attachment whereas others, including fucose and glucose, had no inhibitory effect. In contrast to the findings with the colonic cells, the various pretreatments described above significantly inhibited the adherence process (>90%) only when performed on the bacteria; pretreatments of the ileal cells had no inhibitory effect. These results are summarised in table III.

Table II. Inhibition of the adherence of *E. coli* O124 to guinea pig colonic cells

Compound tested	Percentage adherence*
Fucose (10 mg/ml)	21 ± 3
Glucose (10 mg/ml)	27 ± 6
Mannose (10 mg/ml)	54 ± 3
Rhamnose (10 mg/ml)	97 ± 10
Lactose (10 mg/ml)	98 ± 11
Lipopolysaccharide† (1 mg/ml)	21 ± 1

* Results represent $\bar{M} \pm \text{SEM}$ of ten experiments for fucose, glucose and mannose and three experiments for the other compounds. Adherence is expressed as a percentage of that observed in control tests without the relevant test compound.

† See Methods.

Fig. 2. Inhibition of the adherence of radiolabelled *E. coli* O124 to guinea pig colonic cells by preincubation with unlabelled *E. coli* O128.

Bacterial adherence to intestinal surfaces and to intestinal loops in-vivo

The purpose of these experiments was to determine any correlation between the bacterial adherence to suspension of epithelial cells *in vitro* and the adherence to intestinal surfaces and intestinal loops *in vivo*. *E. coli* O124 avidly adhered to intact colonic surfaces and to colonic loops in a living animal. Quantitative determinations with radiolabelled bacteria showed that the adherence of *E. coli* O128 to the colonic surface was $1.63 \times 10^7 \pm 2 \times 10^6$ bacteria/cm². The number of colonic cells released from the colon was $2.52 \times 10^5 \pm 4 \times 10^4$ cells/cm²; adherence was therefore at a rate of 63 bacteria/cell, similar to the findings *in vitro*. The attachment to the colon *in vivo* was also Ca⁺⁺ dependent, and was inhibited by fucose and glucose (fig. 3). The adherence of *E. coli* O124 to the ileum showed the same characteristics as adherence *in vitro* to ileal cells (data not shown).

Scanning electronmicrographs (fig. 4) showed the marked adherence of *E. coli* O124 to colonic cells. In addition, the bacteria under investigation attached well to the colonic surface, including crypt areas. We found no histological evidence of phagocytosis of the bacteria or of bacteria inside the cells under our experimental conditions.

Discussion

The present results clearly indicate that the

Table III. Comparison of the adherence properties of *E. coli* O124 and *E. coli* O128 to guinea pig intestinal tract

Item	<i>E. coli</i> O124	<i>E. coli</i> O128
Region of adherence	Colon only (especially transverse and descending)	Small and large intestine
Main inhibitory carbohydrate(s)	Fucose, glucose	Mannose
Dependence on Ca ⁺⁺ ions	Yes	Yes
Dependence on pH	Yes (optimum 6.2)	Yes (optimum 6.2)
Pretreatment of intestinal cells	Inhibited adherence (> 90%)	No effect
Pretreatment of bacteria	No effect	Inhibited adherence (> 90%)
Location of the adhesin	On intestinal mucosa	On bacterial surface

Fig. 3. Adherence of *E. coli* O124 to colonic loops in a living animal after incubation for 45 min. The carbohydrates were added together with the radiolabelled bacteria at a final concentration of 10 mg/ml. **Fig. 4.** Scanning electron micrographs of adherence of *E. coli* O124 to guinea pig colonic cells (× 4000).

adherence of the non-fimbriate entero-invasive *E. coli* strain to guinea pig intestinal tract differs from that of the non-invasive ETEC strains studied previously (table III).

The attachment of several non-invasive strains of *E. coli* was shown in this study and in several previous works (Deneke *et al.*, 1979; Gaastra and deGraaf, 1982), to depend on bacterial proteins associated with cell-surface appendages such as pili or fimbriae. These proteins possess specific properties for recognising carbohydrates and are named lectins or adhesins. They are bound to specific receptors, usually carbohydrate moieties, on the intestinal surface. The *E. coli* strain examined in this study bound well to the small intestine, and our findings suggest that a mannose receptor is involved, as has been found in other studies.

On the other hand, the entero-invasive *E. coli* O124 attached only to the colon, mainly its transverse and descending portions. The adherence process was inhibited most effectively by fucose and glucose, which did not affect the attachment of ETEC to the small intestine. Moreover, results of pretreatment studies indicated that the adherence depended on a protein on the host cells of the intestinal surface and not on the bacteria. This protein mediated the attachment of bacteria to the colon by recognising carbohydrate receptors on the bacterial surface. This explains the inhibitory effect of fucose and glucose, as well as the LPS of the bacteria. This adhesin is a carbohydrate-binding protein and is secreted with the intestinal mucus, from which it has been purified and partially characterised by affinity chromatography and sodiumdodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (Mirelman *et al.*, 1985).

The two different patterns of adherence of pathogenic *E. coli* to the gut produce two separate

intestinal syndromes. The ETEC cause an acute watery diarrhoea by colonizing the small intestine and producing an enterotoxin. The invasive *E. coli* usually cause a dysentery-like disease, characterised by high fever, bloody diarrhoea and abdominal cramps. The pathogenesis of the diarrhoea produced by these strains is invasion of the intestinal epithelium, predominantly of the colon. Adherence to the intestine is probably a prerequisite for subsequent enterotoxin action or invasion.

Recently a third mechanism for *E. coli*-associated diarrhoea has been suggested (Ulshen and Rollo, 1980; Rothbaum *et al.*, 1982); this postulates bacterial adherence to the mucosa of the small intestine with disruption of the brush border, but without either enterotoxin production or tissue invasion.

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