An endocytic process in HEP-2 cells induced by enteropathogenic *Escherichia coli*

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Summary. Infection of HEP-2 cells by enteropathogenic *Escherichia coli* (EPEC) was examined by transmission and scanning electronmicroscopy. EPEC strains of serogroups O111:K58 and O55:K59 recently isolated from human patients did not exhibit enterotoxic activity, as judged by the Vero-cell and suckling-mouse assays, or invasive ability as judged by the Sereny test. These strains attached to and penetrated HEP-2 cells. Transmission electronmicroscopy showed bacteria in close contact with cell membranes 15 min after infection; later, intense swelling and budding of membranes and penetration of EPEC into the cell cytoplasm occurred. Intracellular bacteria were enclosed in membrane-bound vacuoles in the cell cytoplasm underlying localised adherence sites observed by light microscopy. Scanning electronmicroscopy showed morphologically altered membranes only at the sites of bacterial attachment. Bacteria inactivated by ultraviolet light were not internalised and cytochalasin B (≥ 10 mg/L) markedly inhibited uptake. These observations suggest that penetration of EPEC into HEP-2 cells occurs by an endocytic process in metabolically active bacteria.

Introduction

Certain serogroups of enteropathogenic *Escherichia coli* (EPEC) are associated with infantile enterocolitis and frequently cause diarrhoeal disease in conditions of poor hygiene (World Health Organization, 1980). Some authors (Konowalchuck *et al.*, 1977; Klipstein *et al.*, 1978; O'Brien *et al.*, 1982) have demonstrated cytotoxic and enterotoxic properties in EPEC, but others (Echeverria *et al.*, 1976; Levine *et al.*, 1978; World Health Organization, 1980) have not.

In naturally infected human patients or experimentally infected animals, adhesion to enterocytes and effacement of microvilli are features of intestinal colonisation by human EPEC (Staley *et al.*, 1969; Ulshen and Rollo, 1980; Rothbaum *et al.*, 1982). Intracellular bacteria are usually limited to the apical cytoplasm of infected enterocytes (Staley *et al.*, 1969; Ulshen and Rollo, 1980; Moon *et al.*, 1983) and are associated with disorganisation of the cytoskeleton and loss of actin filaments (Rothbaum *et al.*, 1982); the intracellular lesions are most readily seen in enterocytes densely colonised by

EPEC (Staley *et al.*, 1969; Moon *et al.*, 1983; Peeters *et al.*, 1984). EPEC strains also adhere to HEP-2 tissue-culture cells, whereas enterotoxigenic *E. coli* and *E. coli* strains from normal individuals do not (Cravioto *et al.*, 1979; Baldini *et al.*, 1983). The bacteria bind to cell surfaces and form microcolonies (Scaletsky *et al.*, 1984). Such local adherence has been suggested as a virulence factor for EPEC (Nataro *et al.*, 1985; Levine *et al.*, 1985; Levine, 1987). We have previously suggested that EPEC infection of HEP-2 cells proceeds through two distinct, but functionally inter-related, steps—bacterial attachment to cell surfaces and internalisation of attached bacteria (Andrade and Santa Rosa, 1986a). Bacterial attachment mediated by non-fimbrial bacterial cell-surface adhesins and putative glycoprotein cell membrane receptors was a prerequisite for internalisation (Andrade and Santa Rosa, 1986b). Knutton *et al.* (1987) confirmed that EPEC adhered intimately to the HEP-2 cell membrane and caused modifications of cell surface architecture that closely resembled EPEC adhesion to intestinal cells.

In the present study we have used scanning and transmission electronmicroscopy to investigate EPEC infection of HEP-2 cells. We also evaluated

the effects of inactivation of EPEC by ultraviolet irradiation, and treatment of host cells with cytochalasin B, a drug which inhibits endocytosis.

Materials and methods

Bacteria

Six human isolates of *E. coli* were used. Some of their characteristics are listed in the table. EPEC serogrouping was performed with OK antisera (Difco). The bacterial strains were stored at -25°C .

Table. Characteristics of the *E. coli* strains used

<i>E. coli</i> strain no.	Serogroup/ serotype	Source	Relevant surface characteristics
54	O111:K58	Sporadic case	non-pilate
68	O55:K59	Sporadic case	non-pilate
386	O111:K58	Outbreak	type-1 pili
3943-67	O111a, b:K58:NM	Stock culture	type-1 pili
ECT-122	O55:K59	Stock culture	type-1 pili
F-2	O?:K?:H4	Normal faeces	non-pilate

Virulence tests

Bacterial invasiveness was tested in albino guinea-pigs by the Sereny test (Sereny, 1955) with bacteria grown on Brain-Heart Infusion Agar (Difco) or in media used for growth of EPEC before infection of cultured cells (see below). Animals were inoculated with 10^8 – 10^{10} cfu or with a loopful of the agar-grown bacteria, and then observed over a period of 72 h for conjunctivitis or corneal clouding and ulceration. Tests for thermolabile enterotoxin (LT) and Vero-toxin (VT) were performed with Vero cells, according to the method of Konowalchuk *et al.* (1977). Production of heat-stable enterotoxin (ST) was evaluated by the suckling-mouse assay (Gomes *et al.*, 1979). Cultures of virulent *Shigella flexneri* (1225/53), LT-positive *E. coli* (H10407), ST-positive *E. coli* (EC3) and VT-positive *E. coli* (H30) were included as controls.

Bacterial growth conditions

Bacteria were grown for 16 h at 37°C in Trypticase Soy Broth (Difco); 500 μl of such a culture was then mixed with 5 ml of antibiotic-free Eagle's Minimal Essential Medium with Earle's salts (MEM: Gibco Laboratories), incubated for 2.5 h at 37°C to yield *c.* 3×10^8 cfu/ml, and then added to cell monolayers.

UV inactivation of bacteria

A 3-ml volume of bacterial culture in MEM was placed in an 85-mm plastic tissue-culture plate and exposed for 5 min to a UV light source (15 W General Electric Germicidal Lamp) with gentle circular agitation. Irradiated bacteria were centrifuged and resuspended in MEM to achieve a concentration of *c.* 10^8 cfu/ml. Viability tests of UV-irradiated cultures yielded no growth.

Cell-culture and infection

The HEP-2 human epithelial cell line CCL 23 (American Type Culture Collection, Rockville, MD, USA) was cultivated in MEM supplemented with calf serum 10% v/v (Instituto Adolfo Lutz, Sao Paulo) gentamicin 50 $\mu\text{g}/\text{ml}$ and amphotericin B 0.25 $\mu\text{g}/\text{ml}$. Cells were grown in glass prescription bottles and were used after 24–48 h when growth was confluent. For scanning electronmicroscopy, monolayers were grown on 8×8 mm glass coverslips.

Cell monolayers were thoroughly washed with Dulbecco's phosphate-buffered saline (PBS-D: NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L, CaCl_2 0.1 g/L, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 g/L; pH 7.2). A 5-ml suspension of bacteria (3×10^8 cfu/ml) in MEM was then added carefully to monolayers. After incubation for 15 min at 37°C (contact stage), monolayers were washed twice with PBS-D prewarmed to 37°C and fresh antibiotic-free MEM was added. Monolayers were then incubated at 37°C for 30 min, 1 h, 4 h or 6 h, with medium changes at 1 and 3 h to minimise extracellular bacterial growth. After the infection period, monolayers were washed twice with PBS-D and fixed with glutaraldehyde 3% v/v. For tests of UV-treated EPEC, bacterial suspensions in MEM were incubated with cell monolayers for 2 h without medium changes.

Treatment of HEP-2 cells with cytochalasin B

Cytochalasin B (Sigma) was prepared as a 1 mg/ml stock solution in dimethyl sulphoxide (Sigma). This stock solution was diluted in MEM to achieve concentrations of 4, 10, 20, 30 and 40 $\mu\text{g}/\text{ml}$ which were added to uninfected cell monolayers and held for 15 min at 37°C . The infection procedure was 2 h, as already described, except that MEM supplemented with cytochalasin B was used and medium was changed at 1 h.

Transmission electronmicroscopy

Infected monolayers were washed with PBS-D and fixed in cold (4°C) glutaraldehyde 3% in 0.05 M sodium cacodylate buffer (pH 7.2) for 1 h. Cells were gently scraped with a rubber policeman and washed thrice with sucrose 9% w/v in 0.05 M cacodylate buffer (pH 7.2). Cell pellets were embedded in Noble Agar (Difco) 1% w/v in 0.85% NaCl and small blocks were cut, fixed in 1% osmium tetroxide, dehydrated in a graded series of

ethanol (50–100%), treated with uranyl acetate 0.5% and embedded in Polylyte 8001 according to the method of Coiro *et al.* (1972). Ultrathin sections were cut on an LKB Ultratome V, stained with aqueous uranyl acetate 1% w/v and lead citrate (Reynolds, 1963) and viewed with a Phillips EM 301 microscope at 60–100 kV.

Scanning electronmicroscopy

After 30 min, infected cell monolayers on 8×8 mm coverslips were gently washed with PBS-D, fixed in glutaraldehyde-cacodylate buffer for 5 min at room temperature and were washed thrice with sucrose-cacodylate buffer. Coverslips were covered with osmium tetroxide 1% and held for 10 min at 25°C, washed thrice with distilled water, dehydrated through ascending concentrations (50–100%) of ethanol and dried by critical point drying. The specimens were then sputter-coated with gold and viewed with a JEOL JSM 25 II scanning electronmicroscope operated at 25 kV.

Results

Virulence tests

None of the *E. coli* strains was positive in tests for heat stable toxin or caused morphological alterations characteristic of thermolabile or Vero toxins in Vero cells. No significant lesions were detected in the Sereny test with organisms grown either in liquid or on agar media.

Infection of HEp-2 cells

Giemsa-stained monolayers of HEp-2 cells infected by EPEC strains 54, 68 and 386, and observed by light microscopy, revealed the characteristic pattern of adhesion designated "localised adherence" in which bacteria were attached in discrete clusters (fig. 1).

Scanning electronmicroscopy of cell monolayers infected for 30 min with the non-pilate EPEC strains 54 and 68 showed bacterial clusters distributed on cell surfaces. Abundant filiform and digitiform projections were observed beneath the sites of bacterial attachment. Regions of plasma membrane around bacterial clusters appeared normal with small microvilli (fig. 2). HEp-2 cells infected with the pilate strain 386 showed isolated bacteria as well as clusters. Membrane budding around attached bacteria and signs of bacterial internalisation were also noted for the isolated bacteria. Most pilate bacteria displayed filiform

Fig. 1. *E. coli* 54, showing "localised adherence" to HEp-2 cells. Giemsa, $\times 870$.

projections binding the bacterial surfaces to the subjacent plasma membrane (fig. 3).

Transmission electronmicroscopy of cells 15 min after addition of non-pilate EPEC strains 54 and 68 showed bacteria separated from the plasma membrane by a gap of $0.01 \mu\text{m}$. At the points of contact, the membrane showed an increase in density and was frequently cupped along the adherent bacterium (fig. 4). At this time, most bacteria were extracellular, but a few were observed to be intracellular or undergoing internalisation. Digitiform projections frequently entangled cell-adherent bacteria (figs. 4 and 5). Ultrastructural signs of cell damage were not detected. Thirty minutes after infection, most bacteria were undergoing internalisation (fig. 5). From 1 to 4 h after infection, penetration of EPEC organisms into the cytoplasm of cells was seen frequently, with individual bacteria enclosed in membrane-bound vacuoles (fig. 6). These vacuoles appeared to fuse as they moved to the perinuclear region giving rise to larger vacuoles containing several bacteria (fig. 7). The major cellular alterations at 2 h and 4 h consisted of intense swelling and budding of plasma membrane beneath and in the immediate neighbourhood of the attached EPEC. Bacteria that appeared to be dividing were observed inside

Fig. 2. Scanning electronmicrograph of HEP-2 cell infected with *E. coli* 54. Bacilli are clumped together. Digitiform projections (arrows) are present adjacent to the aggregates of bacteria. Cell membrane around bacterial clusters appears unaltered (n). Bar, 1.0 μ m.

Fig. 3. Scanning electronmicrograph of HEP-2 cell infected with type-1 pilate *E. coli* 386. Cell-membrane projections arise from regions adjacent to individual bacilli attached to the cell surface. Some bacteria seem to be undergoing internalisation (small arrow). Filiform projections suggesting bacterial pili seem to make contact with the cell surface (large arrow). Bar, 1.0 μ m.

vacuoles and others lay free in the cytoplasm apparently without membrane enclosure (fig. 7).

Infection of HEP-2 cells with type 1-pilate EPEC strain 386 showed similar ultrastructural changes, but more bacteria were attached to cell surfaces.

Most adherent bacteria were closely associated with the plasma membrane. At 6 h after infection, extensive destruction of cell monolayers occurred; the remaining cells were full of intracellular bacteria and were severely damaged.

Fig. 4. Transmission electronmicrograph of HEp-2 cell 15 min after infection with *E. coli* 54. A small gap (0.01 μm) separates closely apposed bacterial and cell surfaces. Terminal web is dense under site of bacterial attachment. Cell membrane forms cups at the base of attached organism. Digitiform projections (d) arise from regions closely adjacent to attachment site and bind to bacterial surface. Bar, 0.2 μm .

Non-EPEC strain F-2 and EPEC stock strains 3943-67 and ECT 122 did not exhibit localised adherence and binding to cell surfaces was rarely found. At the infrequent points of contact between bacterial and cell surfaces, modification of the plasma membrane did not occur. No penetration into cell cytoplasm, induction of alterations to the membrane or other cell damage was observed.

UV irradiation of EPEC

UV irradiated EPEC strains did not penetrate HEp-2 cells during the time of the experiment. A few bacteria were found separated from the

epithelial cell surfaces by a 0.01 μm -gap, but alterations of plasma membrane at the sites of bacterial binding were not seen.

Effect of cytochalasin B on HEp-2 infection

Cytochalasin B at a concentration of 4 $\mu\text{g}/\text{ml}$ did not inhibit internalisation of EPEC by HEp-2 cells. However, a reduction in the number and length of digitiform projections and membrane pedestals beneath the attached bacteria was noted. Concentrations of 10 or 20 $\mu\text{g}/\text{ml}$ caused a substantial reduction in the number of HEp-2 cells with intracellular bacteria and in the number of bacteria internalised; polybacterial vacuoles were rarely found. Cytochalasin concentrations of 30 and 40 $\mu\text{g}/\text{ml}$ almost completely abolished bacterial internalisation. Cells containing isolated bacteria in small vacuoles were occasionally found.

Discussion

These results and earlier findings suggest that infection of HEp-2 cells by EPEC correlates with virulence of recently isolated strains: non-enteropathogenic *E. coli* and EPEC stock cultures did not undergo internalisation even when larger bacterial numbers (10^9 and 10^{10} cfu/ml) and longer incubation periods were used in cell infection assays.

Internalisation of EPEC by HEp-2 cells differs from invasion by *Shigella* spp. or entero-invasive *E. coli*. Thus, despite the use of conditions favourable for bacterial internalisation, none of our EPEC strains showed entero-invasive ability as judged by the Sereny test. Microscopy of stained monolayers of HeLa or Henle cells infected by entero-invasive *E. coli* or *Shigella* spp. show elongated bacteria scattered throughout the cytoplasm after incubation for 5–8 h and bacterial clumps are not found at an early stage of infection (Gerber and Watkins, 1961; Ogawa *et al.*, 1967, 1968; Calabi, 1970). Marked ultrastructural differences also occur. Shortly after infection of cultured cells by *Shigella* spp. (Hale *et al.*, 1979) and entero-invasive *E. coli* (Knutton *et al.*, 1984) a few bacteria attach to cell surfaces or become intracellular, but it is possible to observe unequivocal bacterial penetration only after several hours. Moreover, only minor alterations of the plasma membranes are seen at any time. In contrast, EPEC infection of cultured cells results in a marked early mobilisation of plasma membrane at the points where micro-organisms are intimately attached to the cell surface.

Adherence of EPEC organisms is a prerequisite for bacterial internalisation into HEp-2 cells; non-

Fig. 5. Transmission electronmicrograph of HEP-2 cell 30 min after infection with *E. coli* 54. Marked formation of digitiform projections and membrane folding occurs at or near the bacterial attachment site. Some bacteria are undergoing internalisation. Bar, 0.5 μ m.

Fig. 6. Transmission electronmicrograph of HEP-2 cell 2 h after infection with *E. coli* 68. Intracellular bacteria are enclosed in membrane-bound vacuoles that seem to migrate to the nuclear region. Bacterial vacuoles cause a marked depression on nuclear membrane; n = cell nucleus. Bar, 0.42 μ m.

fimbrial, haemagglutination-negative bacterial adhesins mediate attachment to the cell surface (Andrade and Santa Rosa, 1986a, 1986b). Adherence is specifically blocked by galactose, glucose and rhamnose and even among EPEC strains of

the same serogroup a great diversity of adhesins is anticipated from sugar-inhibition adherence tests (Andrade and Santa Rosa, 1986b). Such EPEC adhesins were called endocytosis-inducer adhesins to emphasise their contribution to bacterial pene-

Fig. 7. Transmission electronmicrograph of HEP-2 cell 4 h after infection with *E. coli* 54. Organisms accumulate in cell cytoplasm beneath attachment site. Large polybacterial vacuoles (pv) and multiplication of intracellular micro-organisms (m) are shown. Some bacteria are in direct contact with cell cytoplasm (*). Small vacuoles (v) suggest cell damage. Bar, 0.54 μ m.

tration into cultured cells (Andrade and Silva, 1987).

Once attached to plasma membrane, viable EPEC were rapidly internalised. The uptake of particulate material by cultured cells occurs by endocytosis, implying active mobilisation of plasma membrane by interaction between actin filaments and myosin, the main components of the cell contractile system. Cytochalasin B hinders the addition of actin molecules to actin filaments leading to their depolymerisation, with inhibition of endocytosis and cell locomotion, membrane ruffling and interference with the formation of microvilli or microspikes (Allison *et al.*, 1971; Alberts *et al.*, 1983). Treatment of HEP-2 cells with cytochalasin B inhibited the formation of digitiform projections and membrane budding and halted internalisation of EPEC without any significant decrease in bacterial attachment to plasma membrane.

Internalisation of EPEC seems to proceed through an endocytic process requiring metabolically active bacteria, since UV-inactivated EPEC bound to but were not internalised by HEP-2 cells. Bacterial viability also plays an essential role in penetration of *Sh. flexneri* and *S. typhimurium* into human epithelial cells (Kihlström and Edebo, 1976; Kihlström and Nilsson, 1977; Hale and Bonventre,

1979). Viable EPEC probably induce some kind of interference in the actin-myosin cell contractile network at the sites of bacterial attachment leading to a local proliferation of cell extensions and membrane budding and infolding. Scanning electronmicroscopy of infected cells showed marked membrane mobilisation only at sites of bacterial attachment.

Small intestinal cells of infants (Ulshen and Rollo, 1980; Rothbaum *et al.*, 1982) or animals (Staley *et al.*, 1969; Moon *et al.*, 1983; Peeters *et al.*, 1984) infected by EPEC show ultrastructural alterations similar to those seen here in infected HEP-2 cells. Furthermore, jejunal enterocytes from infants have specific receptors for EPEC endocytosis-inducer adhesins, since the binding of some EPEC strains to enterocytes is specifically inhibited by carbohydrates that block attachment to and internalisation of the same strains by HEP-2 cells (Andrade and Silva, 1987). Thus, infection of HEP-2 cells by EPEC seems to provide a useful and reliable model for testing EPEC attachment to and penetration of enterocytes.

HEP-2 monolayers infected with strain 386, which exhibits type 1 pili and endocytosis-inducer adhesins, showed more infected cells and more severe cellular degeneration than cell cultures infected with the non-pilate strains 54 and 68.

Scanning electronmicroscopy suggests that pili of strain 386 co-operate in bacterial binding to the cell surface. We showed previously that EPEC with type 1 pili adhered more readily to HeLa and Hep-2 cells (Andrade *et al.*, 1984; Andrade and Santa Rosa, 1986b) and to human enterocytes (Andrade and Silva, 1987) than non-pilate EPEC strains. Hydrophobic properties and the spatial arrangement of type-1 pili could facilitate the initial contact with the cell surface, improving further interaction between endocytosis-inducer adhesins and their

specific receptors, thereby increasing the number of internalised micro-organisms and the severity of cell damage.

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