Adhesion to and invasion of human colon carcinoma Caco-2 cells by *Aeromonas* strains

Y. NISHIKAWA, A. HASE, J. OGAWASARA, S. M. SCOTLAND*, H. R. SMITH* and T. KIMURA

Department of Epidemiology, Osaka City Institute of Public Health and Environmental Sciences, Tennoji, Osaka 543, Japan and * Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

Summary. The enteropathogenicity of *Aeromonas* strains that showed mannose-resistant adhesion to INT407 cells was evaluated by infecting Caco-2 cells and observing them by light and electronmicroscopy. Five of six strains adhered in large numbers to Caco-2 cells in the presence of mannose and caused cytopathic effects. Two strains of *Aeromonas* spp. seemed to invade Caco-2 cells, as membrane-bound bacteria were seen within the cytoplasm of these cells; however, staining by acridine orange-crystal violet appeared to show intracellular fluorescent bacteria in three strains. Fimbriae did not appear to play an important role in adhesion because fimbrial structures were not seen by transmission electronmicroscopy. Adhesion of four strains was inhibited by the addition of L-fucose. The strains were negative in the fluorescence actin staining test, which in enteropathogenic *Escherichia coli* strains correlates with the ability to attach and efface intestinal microvilli. The DNA of the *Aeromonas* strains did not hybridise with the *E. coli eae* and *ipaB* probes, associated with attaching and effacing ability and invasion, respectively. These results give support to the enteropathogenicity of adhesive strains of *Aeromonas* spp., although the mechanisms of adhesion, and possibly invasion, remain to be elucidated.

Introduction

Aeromonas organisms have been recognised as causal agents of human gastrointestinal disease.^{1–3} However, the organisms are autochthonous inhabitants of aquatic environments and are isolated frequently from various foods and environmental specimens.^{4–6} It is not known whether all such strains are pathogenic.

Most strains of *Escherichia coli* are non-pathogenic in the intestine, although some can produce diarrhoea by a number of distinct mechanisms.^{7,8} Most aeromonads might similarly be non-pathogenic in the intestine and only some strains possess virulence factors. Methods for discrimination between isolates of *Aeromonas* spp. that are pathogenic for man, and those from the environment that may be non-pathogenic, are clearly required. Although β -haemolysin production might seem to be a likely pathogenic factor of *Aeromonas* spp.,^{9,10} the failure of haemolytic strains to cause diarrhoea in human volunteers suggests that haemolysin *per se* is not the sole determinant of virulence.¹¹

Adhesion of bacteria to mucosal surfaces has been recognised as an important step in the pathogenesis of most infections in man and animals. Enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae* induce diarrhoea

by elaboration of enterotoxin after adhesion to the intestinal mucosa.¹² Both adhesins and enterotoxins were necessary for production of diarrhoea in volunteers fed orally with ETEC.¹³ Although most strains of *A. hydrophila* and *A. sobria* produce β -haemolysin as a possible enterotoxin, few strains may possess adhesins.⁵

Recently, we found that aeromonads that were adhesive to INT407 cells in the presence of mannose were present at significantly higher levels among strains isolated from faeces than among strains isolated from samples of food and river water.¹⁴ These findings suggest that mannose-resistant adhesion may be important for infection by aeromonads. However, volunteer experiments would be needed to prove this.

The Caco-2 cell line, which was derived from a carcinoma of the colon, exhibits the morphological and functional characteristics of normal small intestinal cells in post-confluent culture.¹⁵ Caco-2 cells are being recognised as a substitute for human intestinal epithelial cells and are used to study the adhesion and invasion of enteric pathogens.^{16–21} Pani-grahi *et al.*²² showed that the adhesion of non-O1 *V. cholerae* to Caco-2 cells correlated with human intestinal colonisation or disease occurrence or both.

In this study, to assess the enteropathogenicity of aeromonads that had shown mannose-resistant adhesion to INT407 cells, Caco-2 cells were infected with the organisms and cytopathogenic effects were ob-

served by scanning and transmission electronmicroscopy. In some experiments, Caco-2 cell cultures of different age were compared since cell differentiation is growth related;¹⁵ adhesion of ETEC and the expression of receptors for diffusely adhering *E. coli* has been reported to be dependent on the differentiation of Caco-2 cells.^{16,19} In addition, the strains were examined by DNA probing for the presence of genes homologous to the *E. coli* attaching and effacing gene *eae* and the invasion gene *ipaB*.^{23,24}

Materials and methods

Bacteria

Four *A. hydrophila* strains (nos. 22, 62, 107 and 189) and two *A. sobria* strains (nos. 21 and 106) were used. These strains were isolated originally from human faeces,⁵ and had shown marked mannose-resistant adherence to INT407 cells.¹⁴ An enteroinvasive *E. coli* strain (E35990, O143:H—) was used as a positive control in *ipaB* hybridisation tests and in studies of cell invasion. An enteropathogenic *E. coli* strain (E2347/69, O127:H6) was used as a positive control in *eae* hybridisation tests.

Epithelial cells

Caco-2 cells were obtained from the American Type Culture Collection (ATCC; HTB37) and cultured according to the method of Finlay and Falkow.¹⁸ Cells were grown in Eagle's Minimal Essential Medium (EMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) with Earle's salts and 2 mM glutamine, supplemented with fetal bovine serum 10% and sodium pyruvate (Flow Laboratories, Irvine) 1% without antibiotics. Transwell filter units (3415; Costar, Cambridge, MA, USA) containing a 0.33-cm² porous filter membrane (3.0-µm pores) and eight-chambered tissue culture slides (Nunc, Naperville, IL, USA) were used to culture Caco-2 cells for the infection experiments for electronmicroscopy and light microscopy, respectively. A trypsinised Caco-2 cell suspension (250 µl) in tissue culture medium was added apically to each transwell unit or to each chamber of the chambered slides. The transwell unit was placed in a well containing 0.5 ml of fresh medium, in a 24-well tissue culture plate. Tissue culture medium was changed twice weekly, and monolayers were used after incubation for > 14 days (37°C, CO₂ 5%) unless otherwise stated. In some experiments, 5-day-old cultures of Caco-2 cells were used for comparison.

Tissue culture adhesion

Adherence tests were performed as described previously.¹⁴ To prepare inocula, the strains were seeded into nutrient broth and incubated statically for 18 h at 30°C. A 200-fold dilution of each culture was made in EMEM supplemented with D-mannose 1%, 2 mM

glutamine and fetal calf serum 2%, and used as inoculum; it contained *c.* 5 × 10⁸ cfu/ml. In some experiments, L-fucose 1% or D-galactose 1% replaced D-mannose. A 250-µl portion of each inoculum was introduced into each chamber or transwell unit and incubated for 30 min at 37°C (the infection period). Unattached bacteria were then removed from the monolayer by three washings *in situ* each with 250 µl of Hanks's Balanced Salts Solution. Then 250 µl of EMEM supplemented with fetal calf serum 2% and D-mannose 1% or L-fucose or D-galactose was added to each chamber. After incubation for 1, 2 or 3 h at 37°C (the multiplication period), the monolayers were washed four times, each time with 250 µl of Hanks's Balanced Salts Solution. The cells on chamber slides were then fixed with methyl alcohol for 5 min and stained for 20 min with Giemsa 10% for light microscopy.

Actin accumulation tests

The fluorescence actin staining test (FAS test) was performed as described previously.²⁵ Briefly, Caco-2 cells were fixed with formaldehyde 3% at the end of an adhesion test. Cells were rendered permeable with Triton X100 0.1% v/v in phosphate-buffered saline (PBS), treated with fluorescein isothiocyanate-phalloidin (Sigma) 5 µg/ml in PBS and examined for the presence of fluorescing accumulations of filamentous actin beneath attached bacteria by consecutive incident light fluorescence and phase-contrast microscopy of the same microscopic fields.

Electronmicroscopy

For electronmicroscopy, Caco-2 cells on transwell units were fixed in phosphate-buffered glutaraldehyde 1% at 4°C. Some samples for transmission electronmicroscopy were fixed in phosphate-buffered glutaraldehyde 1% containing ruthenium red 0.075%. For scanning electronmicroscopy, fixed cells were dehydrated in ascending concentrations of ethanol (25–100%), and critical point dried in liquid CO₂. Specimens were mounted on stubs with silver paint, sputter coated with gold palladium, and examined with a Jeol scanning electronmicroscope (JSM-5300). For transmission electronmicroscopy, samples were post-fixed in cold OsO₄ 1% in 0.1 M phosphate buffer, pH 7.3, for 2 h and then stained with saturated uranyl acetate overnight. Samples were dehydrated in a series of increasing amounts of ethanol and embedded in epoxy resin, then sectioned and stained with lead citrate before examination in a Jeol electronmicroscope (JEM-1200 EXII).

Staining of intracellular bacteria

Acridine orange-crystal violet staining was performed to detect specifically intracellular bacteria by a modification of the method of Miliotis.²⁶ After the

Fig. 1. Light micrographs of Caco-2 cells infected by *A. hydrophila* (strain no. 189). **A**, massive diffuse adhesion can be seen on the cells; **B**, no adhesion is visible in the presence of L-fucose. ($\times 575$).

Fig. 2. Scanning electronmicrographs of Caco-2 monolayers infected by *A. sobria* (strain no. 106). **A**, uninfected monolayer with well-defined brush borders; **B**, Caco-2 cells in early stages of infection, aeromonads interacting with decreased microvilli; **C**, irregularities in the distribution of microvilli can be seen. Bar = 1 μ m.

tissue culture adhesion test, Caco-2 cells were stained with acridine orange 0.01% in Earle's Balanced Salts Solution (Flow Laboratories, Irvine) for 45 s, and counter-stained with crystal violet 0.05% in physiological saline solution for 30 min. Although crystal violet was reported²⁶ to quench the fluorescence of *E. coli* in 45 s, it took 30 min for *Aeromonas* spp. to do so. After the cells were rinsed with Earle's salts solution, slides were viewed under a fluorescence microscope. All infecting bacteria take up the acridine orange and

fluoresce; only intracellular bacteria should be resistant to quenching with crystal violet and remain visible by fluorescent light microscopy.

DNA hybridisation tests

The presence of DNA sequences associated with attaching and effacing lesions was detected with a 1-kb *KpnI-SalI* fragment, the *eae* probe.²³ The probe for invasion sequences was a 1-kb *EcoRI-SalI* fragment

Fig. 3. Transmission electronmicrographs of Caco-2 monolayers infected by *A. hydrophila* (strain no. 62). A, uninfected cell showing well developed microvilli; B, aeromonads adhering to microvilli; C, the grossly damaged microvillous cell surface; D, bacteria enclosed within membrane-bound vacuole inside Caco-2 cell; a bacterium is likely to be in direct contact with cell cytoplasm. Bar = 1 μ m.

derived from plasmid H1 and was kindly provided by Dr Sansonetti, Institut Pasteur.²⁴ Restriction fragments for probes were excised under long wavelength UV from preparative agarose gels. The gel slice was boiled and the DNA was labelled with deoxyadenosine 5'- α -[³⁵S]-thiotriphosphate by the random primer method.²⁷ *Aeromonas* strains were grown overnight at 37°C in nutrient broth. Broth cultures were spotted on to nylon membranes (Hybond N, Amersham) placed on nutrient agar plates, and grown at 37°C for c. 6 h. Bacteria were lysed and their DNA was denatured according to the standard method.²⁸ DNA was fixed to the nylon matrix by baking at 80°C for 2 h. Hybridisation was carried out in a mixture containing formamide 50% v/v, 5 $\times$ SSPE (1 $\times$ SSPE, pH 7.4, is 0.18 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA) and 0.1% each of Ficoll (mol. wt 400 000), polyvinylpyrrolidone, bovine serum albumin and SDS, 0.01 M dithiothreitol and 100 μ g denatured salmon sperm DNA/ml. After washing for 2 h in 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl, 1 mM EDTA and SDS 0.1%, nylon filters were incubated for 4 h at 42°C in hybridisation solution without labelled probe. They were then transferred to fresh hybridisation solution containing 10⁶–10⁷ cpm heat-denatured probe DNA and incubated overnight at 42°C. The filters were then washed in: (i) four changes (10 min each) of 2 $\times$ SSC

containing SDS 0.1% at room temperature (1 $\times$ SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0); (ii) 1 $\times$ SSC containing SDS 0.1% at 68°C for 2 h; (iii) 0.2 $\times$ SSC containing SDS 0.1% for 1 h at 68°C. The filters were allowed to dry at room temperature and were then exposed to X-ray film.

Results

All six strains of *Aeromonas* spp. showed mannose-resistant diffuse adhesion to Caco-2 cells (fig. 1A) irrespective of the age of the monolayers, although the adhesion of one strain (no. 21) was less than had been seen with INT407 cells. The adherence of four strains (nos. 62, 106, 107 and 189) was inhibited by the addition of L-fucose to the tissue culture medium (fig. 1B), but was unaffected by the addition of D-galactose. No accumulation of filamentous actin was observed in FAS tests in spite of massive adhesion of aeromonads.

Electronmicroscopy showed that Caco-2 cells produced a well-defined brush border with microvilli at their apical surfaces (figs. 2A and 3A). As expected from the light microscopic studies, each strain showed adherence to Caco-2 cells. After a 1-h multiplication period, the organisms could be seen adhering to intact microvilli. On some cells, there was damage to the brush border and signs of disruption and irregularities

Fig. 4. Acridine orange-crystal violet staining of Caco-2 cells, **A**, fluorescent intracellular organisms of enteroinvasive *E. coli* E35990; **B**, *A. hydrophila* (strain no. 62) showing no fluorescent bacteria in 5-day-old culture of Caco-2 cells; **C**, numbers of fluorescent aeromonads (strain no. 62) in 14-day-old culture of Caco-2 cells. ($\times 575$).

in the distribution of microvilli were observed (figs. 2B and C). After a 2-h multiplication period, extensive adhesion was observed (fig. 3B). As the infection continued (2–3 h), disruption of the cells became more apparent (fig. 3C), and the cells detached from the support. Some aeromonads (strain nos. 62 and 106) were found in membrane-bound inclusions within Caco-2 cells (fig. 3D). Occasionally, the membrane of the vacuole was difficult to discern; therefore, it was not clear whether these aeromonads always remained within membrane-bound vacuoles after invasion.

Intracellular fluorescent enteroinvasive *E. coli* were clearly seen by the acridine orange-crystal violet staining method (fig. 4A). Although no fluorescent aeromonads were observed in experiments with 5-day-old cultures of Caco-2 cells by the acridine orange method (fig. 4B), three strains of *Aeromonas* spp. (nos. 22, 62 and 106) showed numbers of fluorescent bacteria in 14-day-old cultures of Caco-2 cells (fig. 4C).

None of the *Aeromonas* strains showed positive reactions in hybridisation tests with the *eae* or *ipaB* probes.

Discussion

Adhesion of bacteria to mucosal surfaces has been recognised as an important process in the pathogenesis

of most infections in man and animals. We had previously found that aeromonads which showed mannose-resistant adhesion to INT407 cells were present at significantly higher levels among strains isolated from faecal specimens than among strains isolated from samples of food or river water.¹⁴ Current experiments revealed that *Aeromonas* strains that had shown mannose-resistant adhesion to INT407 cells were also able to adhere to Caco-2 cells in a mannose-resistant manner. Furthermore, it appeared that some *Aeromonas* strains could be invasive for Caco-2 cells. Morris *et al.* showed that adherence and invasion of non-O1 *V. cholerae* strains to Caco-2 cells correlated with human and rabbit enteropathogenicity.^{22, 29, 30} Although diarrhoeagenicity for man or rabbits of the *Aeromonas* strains used in our study remains to be confirmed, the adhesion to and invasion of Caco-2 cells was as extensive as that of non-O1 *V. cholerae*. This, combined with the observation that these adhesive aeromonads were detected more frequently in faecal specimens than environmental specimens,¹⁴ suggests that these organisms are enteropathogenic.

Previously we observed the presence of a few supple and curvilinear fimbriae on two strains (nos. 106 and 189).¹⁴ However, it seems unlikely that fimbriae played an important role in Caco-2 cell adhesion. Fimbria-like structures were not observed between the aéro-

monads and Caco-2 cells. Moreover, the organisms were seen close to the microvilli, in contrast to the more distant adherence given by enterotoxigenic *E. coli* possessing fimbrial colonisation factor antigens.³¹ A non-O1 *V. cholerae* strain that was Caco-2 adhesive and diarrhoeagenic in human volunteer studies did not produce fimbriae.²² Non-fimbrial adhesive factors such as outer-membrane proteins might be involved in the adhesion.^{32, 33} Fucose inhibited the adhesion of four strains (nos. 62, 106, 107 and 189) to both Caco-2 cells and INT407 cells, suggesting that receptors on the cell surfaces may involve this carbohydrate. By contrast, two other strains may recognise other substances as receptors because their adhesion was unaffected by the sugars tested.

It has been reported that there is a direct correlation between the isolation of *Aeromonas* strains from dysentery-like illness and the ability of the strains to invade HEp-2 cells.^{34, 35} However, a non-O1 *V. cholerae* strain, which was invasive to Caco-2 cells²² and rabbit intestinal epithelium,³⁰ caused diarrhoea but not dysentery in human volunteers.²⁹ Invasion of cells by two strains of *Aeromonas* spp. (nos. 62 and 106) was seen by electronmicroscopy, and these strains also appeared as intracellular fluorescent bacteria in acridine orange-crystal violet staining experiments. Another strain (no. 22) appeared to be invasive by the acridine orange method but this could not be confirmed by electronmicroscopy. Although the enteroinvasive *E. coli* strain could be detected by acridine orange-crystal violet staining to be within Caco-2 cells irrespective of their age, the invasive strains of *Aero-*

monas spp. were not seen within 5-day-old monolayers of Caco-2 cells. The limited invasive ability of *Aeromonas* strains observed in our current experiments suggests that their invasive mechanism may differ from that of enteroinvasive *E. coli* or *Shigella* organisms.

Enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* cause loss of intestinal epithelial cell microvilli and adhere to the cell membrane intimately: the bacteria are partially surrounded by cup-like projections of the apical enterocyte membrane. Knutton *et al.*²⁵ developed the fluorescence actin staining (FAS) assay to screen bacteria for the ability to cause attaching and effacing lesions which is based upon the formation of filamentous actin beneath adhering bacteria. However, all the *Aeromonas* strains showed a negative reaction in FAS tests and hybridisation with the DNA probes for the *E. coli eae* or *ipaB* genes, which are associated with attaching and effacing effects and invasion, respectively.^{23, 24} The negative results by *eae*-probe are concordant with our electronmicroscopic observations, since the latter showed disruption of the cells but no dense concentrations of microfilaments or cup-like projections of the cell membrane beneath adhering bacteria. Further investigations are in progress to confirm the enteropathogenicity and clarify the mechanisms of adhesion because the adhesion to Caco-2 cells does support the diarrhoeagenicity of the *Aeromonas* organisms.

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