Surface properties of *Yersinia* species and epithelial cell interactions *in vitro* by a method measuring total associated, attached and intracellular bacteria

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Summary. A procedure was developed for enumeration of total associated, attached and intracellular bacteria after interaction of *Yersinia* spp. with epithelial cells *in vitro*. Isogenic cultures of *Y. enterocolitica* grown at 25°C had greater affinity for epithelial cells (Henle, HeLa and Vero) than for polystyrene, and they invaded the cells. *Y. kristensenii* and *Y. intermedia* showed less attachment to either surface and were non-invasive. The degree of attachment to cells and invasion by *Y. enterocolitica* was related to number of bacteria added and interaction time, whereas attachment to polystyrene occurred rapidly and did not change. *Y. enterocolitica* was more hydrophobic when grown at 35°C than at 25°C according to partitioning in a biphasic dextran-polyethylene glycol system, and attached strongly to both polystyrene and epithelial-cell monolayers. *Y. kristensenii* grown at 25°C was also hydrophobic but did not have the same attachment properties. *Y. kristensenii* and *Y. intermedia* showed slightly reduced electrostatic interactions with the anion exchangers DEAE-Sepharose and DEAE-Trisacryl. Attachment of *Y. enterocolitica* to epithelial cells probably involves non-specific surface properties that are not entirely explicable by hydrophobic and electrostatic interactions, whereas invasion of epithelial cells appears to resemble "receptor-mediated endocytosis".

Introduction

Animal cells in tissue cultures have frequently been used in in-vitro assays to examine the adherence and invasive properties of pathogenic bacteria including *Yersinia enterocolitica*. Measurement of the association of bacteria with animal cells *in vitro* has most commonly been made by microscopy of stained monolayers (Hartley *et al.*, 1978; Hackney *et al.*, 1980; Tavendale *et al.*, 1983). Some procedures have divided the bacterial-animal cell interaction into two phases, the infection phase and the intracellular growth phase (Lee *et al.*, 1977; Une, 1977; Une *et al.*, 1977; Pedersen *et al.*, 1979; Okamoto *et al.*, 1980; Soemitro *et al.*, 1981), a consideration that appears unnecessary with *Y. enterocolitica* because it does not multiply intracellularly (Devenish and Schiemann, 1981). Bacteria that do not associate with the animal cells during the interaction period are usually removed by repeated washing (Mäki *et al.*, 1978; Portnoy *et al.*, 1981; Vosbeck and Huber, 1982; Vosbeck *et al.*, 1982; Mintz *et al.*, 1983) but density-gradient

centrifugation has also been used for this purpose (Izhar *et al.*, 1982). If only intracellular bacteria are of interest, attached bacteria that remain after washing are destroyed by addition of an antibiotic (Une, 1977; Une *et al.*, 1977; Pedersen *et al.*, 1979; Soemitro *et al.*, 1981; Schiemann and Devenish, 1982; Okamoto *et al.*, 1984) but a bacteriophage has also been used (Shaw *et al.*, 1983). Some investigators have attempted to improve the identification of the intra- or extra-cellular location of bacteria by microscopy by the use of immunofluorescent staining (Kihlstrom, 1977; Hale and Bonventre, 1979; Jones *et al.*, 1981) or a combination of differential interference contrast and ultraviolet incident light microscopy (Bukholm *et al.*, 1982). Other techniques used for enumeration of cell-associated bacteria have used radiolabelled bacteria (Vosbeck *et al.*, 1982; Schiemann and Swanz, 1985a), selective incorporation of radiolabel by intracellular bacteria (McCoubrey and Howard, 1981), and plate counts of intracellular bacteria that have survived antibiotic destruction and are released by cell lysis (Devenish and Schiemann, 1981; Bhogale *et al.*, 1983; Brunius and Bölin, 1983; Okamura *et al.*, 1983).

The attachment of some enteropathogenic bacteria to epithelial cells takes place through specific adhesin (ligand)-receptors (Fletcher, 1980; Beachey, 1981). For example, some strains of enterotoxigenic *Escherichia coli* bear mannose-resistant haemagglutinating fimbrial adhesins called colonisation factors (CFA/I and CFA/II) (Evans and Evans, 1978; Evans *et al.*, 1975 and 1978) that are putatively specific for receptors on the epithelial cells (Lindahl *et al.*, 1982). Fimbriae are not, however, responsible for epithelial cell attachment by all enteropathogenic bacteria (O'Hanley and Cantey, 1978; Jann *et al.*, 1981; Izhar *et al.*, 1982; Cohen *et al.*, 1985; Deneke *et al.*, 1985) including *Y. enterocolitica* (Old and Robertson, 1981). There is also evidence that epithelial-cell association results from nonspecific physicochemical interactions such as surface charge and hydrophobicity (Perers *et al.*, 1977; Magnusson *et al.*, 1980; Wadstrom *et al.*, 1980; Faris *et al.*, 1981; Edebo *et al.*, 1983) and that fimbriae only promote epithelial-cell association by overcoming electrical repulsion forces (Heckels *et al.*, 1976) and increasing hydrophobicity (Smyth *et al.*, 1978; Lindahl *et al.*, 1981; Faris *et al.*, 1982 and 1983; Kihlstrom and Magnusson, 1983; Honda *et al.*, 1984). The mechanism involved in bacterial invasion (i.e., penetration, infection, internalisation) is less well understood. However, recent studies with *Shigella* and enteroinvasive *E. coli*, in which invasion is plasmid-mediated (Sansone *et al.*, 1982; Silva *et al.*, 1982), indicate that unique outer-membrane proteins may be involved (Maurelli *et al.*, 1985).

In a previous paper (Schiemann and Swanz, 1985a), we reported that strains of *Y. enterocolitica* that were capable of associating with Henle 407 epithelial cells were hydrophobic as judged by attachment to phenyl-Sepharose and by partitioning in hexadecane. These hydrophobic strains did not, however, aggregate in ammonium sulphate, another test of hydrophobicity (Lindahl *et al.*, 1981). In the same study, two strains of *Y. enterocolitica*, two of *Y. kristensenii*, and one of *Y. intermedia* did not associate with epithelial cells but showed evidence of hydrophobicity, indicating that hydrophobicity alone did not account for epithelial cell association by *Yersinia* spp.

For this study, we selected one strain of epithelial cell association-positive *Y. enterocolitica*, one epithelial cell association-negative and hydrophobic strain of *Y. kristensenii*, and an epithelial cell association-negative and non-hydrophobic strain of *Y. intermedia* for further examinations of surface properties. *Y. enterocolitica* was examined for surface properties after growth at 25°C and 35°C

(the higher temperature reportedly changing surface properties such as hydrophobicity), and for adherence to epithelial cells (Vesikari *et al.*, 1981; Heesemann *et al.*, 1984) with isogenic cultures that lacked the virulence plasmid (Gemski *et al.*, 1980; Laird and Cavanaugh, 1980) that has been associated with changes in surface properties (Kapperud and Lassen, 1983; Skurnik *et al.*, 1984; Kapperud *et al.*, 1985a and b; Lachica and Zink, 1984; Lachica *et al.*, 1984). We also describe in this paper a procedure for discrimination between total associated, attached, and intracellular bacteria after interaction with epithelial-cell monolayers.

Materials and methods

Bacteria

The principal test strain in this study was a human faecal isolate of *Y. enterocolitica* serotype O:5, 27 (E641). This culture was autoagglutinable and calcium-dependent at 35°C, representing indirect markers of the Vwa virulence plasmid, and had a biochemical profile typical of pathogenic strains of *Y. enterocolitica* (Schiemann and Devenish, 1982). An isogenic culture that lacked autoagglutination and calcium-dependence was obtained from a Brain Heart Infusion (BHI) broth culture grown at 35°C by plating on magnesium oxalate (MOX) agar and incubating at 35°C (Gemski *et al.*, 1980). The presence and absence of a plasmid of mol. wt (42–48) × 10⁶ in these isogenic cultures was further confirmed by agarose gel electrophoresis.

Other test strains included *Y. kristensenii* serotype O:12, 26 (E709), originally isolated from human faeces, and *Y. intermedia* (serotype not determined) (E814), originally isolated from pork. These species are nonpathogenic according to laboratory markers of virulence in *Yersinia* spp.

Stock cultures were maintained in peptone-glycerol at –20°C and subcultured on Trypticase Soy-0.6% Yeast Extract (TSY) agar and incubated at room temperature (22–25°C) for 48 h before being used immediately in experiments or subcultured again for radiolabelling.

Radiolabelling of bacteria

Test strains were subcultured at a density of about 10⁵ bacteria/ml in a broth medium (BSYE) containing yeast extract 0.1%, (NH₄)₂SO₄ 0.1%, MgSO₄·7H₂O 0.001%, CaCl₂ 0.001%, NaCl 0.1%, and [³⁵S]methionine 10 µCi/ml (pH 7.6) and incubated at room temperature on a drum roller for 48 h. This method provided an average specific activity of 88 bacteria per disintegration/min (dpm) (n=23) which varied primarily with the specific activity of the radiolabel.

Tissue cultures

Henle 407 intestinal epithelial cells (ATCC CCL-6) were maintained in monolayers in Eagle's basal medium with Hanks's salts and fetal bovine serum (FBS) 15% (BME), penicillin 50 IU/ml and streptomycin 50 µg/ml incubated at 37°C in an atmosphere of air + CO₂ 5%. Tissue cultures were discarded after eight passages. Cells were recovered from monolayers by trypsinisation and resuspended in antibiotic-free BME. The cell density was adjusted to 3.25×10^5 cells/ml and 1.0 ml was added to each well in a 24-well tissue-culture plate. Plates were incubated for 48 h when the monolayer was at least 90% confluent. One set of three control wells on each plate received medium and no cells to permit measurement of bacterial attachment to polystyrene. One of three sets of three wells with cells received, 18 h before use, 5 µl of cytochalasin B (Sigma Chemical Company) dissolved in DMSO to inhibit bacterial internalisation and provide a measure of attached bacteria only.

Bacteria-epithelial cell interactions

Radiolabelled bacteria were harvested from BSYE cultures by centrifugation (2800 *g* for 20 min) and resuspended in bovine serum albumin (BSA) 0.1% in Dulbecco's phosphate-buffered saline without calcium (A-DPBS) containing cycloheximide 0.01%. Cycloheximide reduced to a minimum uptake by the epithelial cells of whatever free radiolabel remained in the bacterial suspension. The density of the bacterial suspension was adjusted spectrophotometrically to 5×10^8 /ml; this was confirmed by plate counts ($\bar{X} = 5.5 \times 10^8 \pm 6.1 \times 10^7$ /ml; *n* = 11). The radioactivity of the bacteria was determined by adding 100 µl of the suspension to 10 ml of Aquasol (New England Nuclear, Boston, MA, USA) placed in a scintillation counter (Tri-Carb 460 CD Liquid Scintillation System, Packard Instrument Company, Inc., Downers Grove, IL, USA). Counts/min were converted to disintegrations/min (dpm) which were used to determine the specific activity of the bacteria. The specific activity was used later to convert radioactivity measurements of epithelial cell-associated bacteria to numbers of bacteria.

All wells in the tissue-culture plate were washed twice with A-DPBS and then treated with BSA 1.0% in DPBS to reduce non-specific binding of bacteria to polystyrene. The BSA-DPBS was removed after 1 h and 0.5 ml of the bacterial suspension was added to each well. The bacteria were removed after 2 h and the wells were washed five times with A-DPBS. One untreated set of monolayers, the cytochalasin-treated set, and the blank wells were then treated with 0.5 ml of a lysis buffer (sodium lauryl sulphate 4%, 10 mM EDTA, 50 mM Tris, pH 12.4). The contents of each well plus one rinse with the same buffer were transferred to 10 ml of Aquasol for measurement of radioactivity. The remaining set of monolayers was washed and then treated with gentamicin (100 µg/ml in A-DPBS) for 1 h. These monolayers were then washed five times with A-DPBS and treated with N-lauroylsarcosine (sarcosine) (Sigma) 1% in NaCl 0.85%. The

bacteria recovered from these wells were measured by plate counts. Counts based on radioactivity measurements and those based on plate counts were converted to numbers of bacteria (or radioactivity in dpm) per monolayer or blank well.

This assay system provided four separate counts: (i) bacteria attached to blank wells, representing non-specific attachment to polystyrene or background; (ii) extracellular attached and intracellular bacteria, representing total associated bacteria; (iii) extracellular bacteria on cytochalasin-treated monolayers, representing attached bacteria only; and (iv) bacteria surviving gentamicin treatment, representing intracellular bacteria. When a monolayer was present, the number of bacteria also represented a certain amount of non-specific association with exposed polystyrene. These counts were, therefore, in most cases adjusted by subtracting the background measurement obtained with blank wells. This was, of course, an over-compensation because the area of exposed polystyrene in wells containing monolayers was less than that in the blank wells used as background. Nevertheless, this adjustment was a means of expressing the relative affinity of the bacteria for epithelial cells compared with that for polystyrene under the same conditions.

Partitioning in dextran-polyethylene glycol

A dextran-polyethylene glycol two-phase system was prepared according to procedures described by Magnusson and Johansson (1977) and Magnusson *et al.* (1977). Stock solutions of dextran T2000 (Pharmacia) 20% w/w and polyethylene glycol (PEG) (Sigma) 20% w/w were prepared in 0.1 M Tris buffer (pH 7.0). PEG 11 g and dextran 15.5 g were mixed with 23.5 g of 0.03 M Tris buffer (pH 7.0) and the mixture was held overnight at 4°C. Two ml of the top phase (PEG-rich) and 2.0 ml of the bottom phase (dextran-rich) were added to an acid-washed glass vial. Bacteria (100 µl) washed from TSY agar cultures and suspended in saline were added to the vial and mixed by gentle inversion 20 times. Large bubbles were broken with a hot inoculating needle. After holding the vial at room temperature for 45 min, 1.0 ml samples of the top and bottom phases were removed, diluted 1 in 10 in saline, and the absorbance was read at 540 nm against a saline blank. The remaining 2.0 ml in the vial were mixed and then diluted 1 in 10 for measurement of absorbance. This fraction was designated the interphase.

Adsorption to DEAE-Sepharose

Columns of DEAE-Sepharose CL 6B and Sepharose CL 6B (Pharmacia) 25–30 mm in height were prepared in glass wool-plugged pasteur pipettes. The columns were equilibrated by repeated washing with either 0.01 M Tris (pH 7.9) or 0.01 M acetate (pH 3.9) buffers. Bacteria were washed from TSY agar cultures in saline, divided into two aliquots, recovered by centrifugation and resus-

pended in buffers (pH 3.9 and 7.9). After holding at room temperature for 1 h, 100 µl of a bacterial suspension was added to the column previously equilibrated with the same buffer. Elution was accomplished by adding 5.0 ml of the same buffer and the absorbance of the eluate was measured at 540 nm against a buffer blank. The results were expressed as decimal decrease in absorbance of the original suspension of bacteria when diluted in 5.0 ml of buffer.

Fractionation on Trisacryl M DEAE

Trisacryl M DEAE (Pharmacia) was equilibrated by repeated washing with 0.01 M Tris buffer (pH 7.9) and used to prepare a column (1 × 10 cm). Bacteria were washed from TSY agar cultures in saline, recovered by centrifugation, washed twice with saline and finally suspended in 0.01 M Tris buffer (pH 7.9). The bacteria were added to the column followed by washing with 30 ml of buffer. The column was then eluted with a

sodium chloride gradient from zero to 1.0 M in 0.01 M Tris buffer (pH 7.9). Elution of the bacteria was followed by monitoring absorbance at 280 nm with a strip chart recorder.

Results

Validation of tissue culture plate method

Interpretation of the results provided by the tissue culture plate method used to measure the interaction of *Yersinia* strains with epithelial cells *in vitro* is explained in table I. The validity of this method was evaluated by plate counts on composites of three wells (monolayers or blanks) from three experiments (table II). The average sum of attached and internalised bacteria was 90.1% of the total associated bacteria. A recovery of <100% probably resulted from over-compensation by subtraction of

Table I. Interpretation of bacterial counts obtained from tissue culture plate method

Monolayer	Treatment		Count represents bacteria
	before addition of bacteria	after addition of bacteria	
B (no cells)			nonspecifically attached to polystyrene (background)
T			attached + intracellular + background (total)
I		gentamicin	intracellular
A	cytochalasin		attached + background
S	cytochalasin	gentamicin	survivors of attached + background

Table II. Validation of tissue culture plate method for measuring associated, attached and intracellular bacteria

Determination	Percentage count			
	Experiment 1	Experiment 2	Experiment 3	Mean
Intracellular bacteria [I/(T-B) × 100]	9.3	12.1	14.1	11.8
Attached bacteria [(A-B)/(T-B) × 100]	69.6	84.0	81.2	78.3
Attached + intracellular bacteria [sum of above]	78.9	96.1	95.3	90.1
Attached bacteria destroyed by gentamicin [(A-S)/A × 100]	95.2	97.4	99.1	97.2

background (i.e., counts on blank wells) and some inhibition of attachment to cytochalasin-treated monolayers. Nevertheless, the results are sufficient to conclude that the method was reliable for measurement of intracellular and attached bacteria. The extracellular location of the bacteria on cytochalasin-treated monolayers was confirmed by finding an average of 97.2% killing with gentamicin. As the data indicate, nearly 12% of the total associated bacteria were intracellular; therefore, total destruction of extracellular bacteria by gentamicin, which is impossible to achieve in 1 h, would be represented by an 88% destruction if the bacteria were divided between extracellular and intracellular locations in these proportions. The difference between 88 and 98% represents nearly a log difference in number of bacteria; therefore, it seems reasonable to conclude that most of the bacteria on cytochalasin-treated monolayers were extracellular.

Multiplicity and interaction time

The adjusted suspension of *Y. enterocolitica* was added to monolayers and blank wells undiluted and at three dilutions to observe the effect of multiplicity,

Fig. 1. Relationship of attachment to polystyrene (background dpm, □), total association (dpm, ▨), attached (dpm, ■) and internalised (number, ▩) bacteria to relative number of *Y. enterocolitica* added per well or monolayer of Henle 407 epithelial cells.

ity, i.e., ratio of bacteria to epithelial cells. The results showed that association increased with the number of bacteria added and that there was no apparent saturation within the range examined (fig. 1).

Bacterial association and attachment to the cell monolayers began almost immediately and increased at a nearly linear rate during the 2-h reaction period (fig. 2). Intracellular bacteria in numbers exceeding background were not detectable until 30 min and then increased sharply without levelling off during the 2-h reaction period. In contrast, attachment to polystyrene appeared to reach a maximum after 30 min.

Tissue culture cell line

The interaction of *Y. enterocolitica* with three different continuous cell lines was examined in two experiments. The cell number used to establish the

Fig. 2. A time sequence of attachment to polystyrene (○), total association (●), attachment (×) and internalisation by Henle 407 epithelial cells (△) of *Y. enterocolitica*. Attachment and total association measured in dpm and internalised bacteria as number/monolayer.

Table III. The effect of tissue culture cell line on association by *Y. enterocolitica*

Experiment number	Cell line	Radioactivity (dpm)*			Number of intracellular bacteria†
		Background	Associated	Attached	
1	Henle	7130 (±588)	70 800 (±913)	59 600 (±4200)	8.57 × 10 ⁴
	Vero	5160 (±602)	106 600 (±1910)	78 800 (±1910)	7.10 × 10 ⁵
2	Henle	11 260 (±759)	65 580 (±1340)	ND	3.83 × 10 ⁵
	HeLa	7381 (±1690)	99 620 (±10 000)	ND	8.20 × 10 ⁵

* Mean ± SD (n = 3).

† Plate count on composite of three determinations.

ND, not done.

monolayer was standardised by counts of viable cells identified by trypan blue exclusion. Bacterial association with Vero and HeLa cells was slightly greater than with Henle cells; Vero cells appeared particularly efficient at internalisation of the bacteria (table III).

Isogenic cultures

An isogenic culture of *Y. enterocolitica* that was autoagglutination-negative and calcium-independent at 35°C and lacked the virulence plasmid according to agarose electrophoresis, showed only slightly reduced association with Henle 407 epithelial cells (table IV).

Growth temperature and bacterial species

Y. enterocolitica grown at 35°C showed a higher degree of attachment to polystyrene than cultures

Table IV. Epithelial cell association by isogenic cultures of *Y. enterocolitica*

Location of bacteria	Percentage of bacteria added per monolayer*		Percentage decrease with plasmid loss
	Plasmid-positive	Plasmid-negative	
Associated	1.36/2.33†	0.417/0.672	69/71
Attached	1.05/1.69	0.146/0.258	86/85
Internalised	0.079/0.084	0.012/0.011	85/87

* Average of three determinations; all values were adjusted for background attachment to polystyrene.

† Experiment 1/Experiment 2.

grown at 25°C (table V). This background for bacteria grown at 35°C represented nearly 50% of the number of bacteria associated with cell monolayers compared with <10% for bacteria grown at 25°C.

Neither *Y. kristensenii* nor *Y. intermedia* associated with epithelial cells to the same degree as *Y. enterocolitica* (table V). The difference between these cultures was greater when the comparison was based on the number of intracellular bacteria (i.e., 0.2–0.4% of the number of *Y. enterocolitica*).

Partitioning in dextran-PEG

Isogenic cultures of *Y. enterocolitica* grown at 25°C showed very similar partitioning in dextran-PEG with the largest proportion of the bacteria in the top and middle phases (fig. 3). *Y. enterocolitica* (plasmid-positive) grown at 35°C showed a different pattern; there was an increased proportion of bacteria in the dextran-rich bottom phase. *Y. kristensenii* and *Y. intermedia* showed entirely different partitioning patterns from each other; *Y. kristensenii* was concentrated to a greater degree in the bottom phase.

Adsorption on to DEAE-Sephrose

The adsorption of epithelial cell association-positive cultures of *Y. enterocolitica* on to DEAE-Sephrose was complete at pH 3.9 and only slightly less at pH 7.9 regardless of growth temperature or plasmid presence (fig. 4). The adsorption of epithelial cell association-negative cultures of *Y. kristensenii* and *Y. intermedia* was only slightly less at each pH. There was, however, an unusual difference

Table V. Influence of growth temperature and species on Henle 407 epithelial cell association by *Yersinia*

Species (serotype)	Growth tempera- ture (°C)	Percentage of bacteria added*					
		back- ground	associated		attached		interna- lised
			total	less back- ground	total	less back- ground	
<i>Y. enterocolitica</i> (O:5, 27)	25	0.109 (6.06)†	2.11	2.00	1.84	1.73	0.108
	35	6.38 (44.9)	7.96	1.58	9.68	3.30	0.074
<i>Y. kristensenii</i> (O:12, 26)	25	0.035 (3.37)	0.473	0.438	0.188	0.153	0.0002
<i>Y. intermedia</i> (not determined)	25	0.013 (2.97)	0.433	0.420	0.150	0.137	0.0004

* Average of three determinations.

† Percentage of number of associated bacteria.

between these cultures in adsorption to Sepharose at pH 3.9 which was not observed at the higher pH. At the lower pH, the epithelial cell association-positive cultures adsorbed strongly to Sepharose whereas the negative cultures did not.

A major concern with passage of bacteria through gel columns is that removal of such a large particle

may occur by physical filtration alone. This was not the case for Sepharose at pH 7.9; nearly complete passage occurred at low ionic strength. Furthermore, the interaction with DEAE-Sepharose and DEAE-Trisacryl appeared to be specific because the bacteria could be eluted at certain concentrations of NaCl (see below).

Fig. 3. Relative degree of partitioning of plasmid-positive (+) *Y. enterocolitica* (E641) grown at 25°C and 35°C, and plasmid-negative (-) *Y. enterocolitica* (E641) grown at 25°C, and *Y. kristensenii* (E709) and *Y. intermedia* (E814) grown at 25°C, in a PEG-dextran biphasic system.

Fig. 4. Adsorption of plasmid-positive (+) *Y. enterocolitica* (E641) grown at 25°C and 35°C and plasmid-negative (-) *Y. enterocolitica* (E641) grown at 25°C, and *Y. kristensenii* (E709) and *Y. intermedia* (E814) grown at 25°C, on to DEAE-Sepharose (■) and Sepharose (□) at pH 3.9 and 7.9. A decimal decrease of 1.0 in A_{540} represents total adsorption.

Fig. 5. Elution of plasmid-positive *Y. enterocolitica* grown at 25°C from DEAE-Trisacryl by a NaCl gradient. Elution peaks measured at 280 nm occur at 0.16 M and 0.46 M NaCl.

Elution from DEAE-Trisacryl

Elution of plasmid-positive 25°C-grown *Y. enterocolitica* adsorbed to DEAE-Trisacryl by a NaCl continuous gradient produced two peaks, one at 0.16 M and the other at 0.46 M NaCl (fig. 5). Bacteria adsorbed to DEAE-cellulose produced the same elution profile as those adsorbed to DEAE-Trisacryl. Growth of the bacteria for 24 instead of 48 h, when most of the bacteria were thought to be in the logarithmic growth phase, produced identical elution patterns (data not shown). Cloning bacteria from the second elution peak and testing after subculture resulted in the same elution profile. The plasmid-negative isogenic culture of *Y. enterocolitica* produced a similar elution profile with peaks at

0.20 M and 0.43 M NaCl (data not shown). Agar- and broth-grown *Y. enterocolitica* showed equivalent association with epithelial cells *in vitro* but broth-grown bacteria produced two additional elution peaks. Bacteria grown on TSY agar at 35°C appeared to react non-specifically with DEAE-Trisacryl with gradual elution between 0.25 M and 0.70 M NaCl (fig. 6). Epithelial cell association-negative cultures of *Y. kristensenii* and *Y. intermedia* showed similar NaCl gradient elution profiles which differed from those for *Y. enterocolitica*. For these species, the majority of bacteria eluted at 0.07–0.11 M NaCl without any equally prominent peak at higher concentrations of NaCl (figs. 7 and 8).

Fig. 6. Elution of plasmid-positive *Y. enterocolitica* grown at 35°C from DEAE-Trisacryl by a NaCl gradient.

Fig. 7. Elution of *Y. kristensenii* grown at 25°C from DEAE-Trisacryl by a NaCl gradient. The first peak occurs at 0.07 M NaCl.

Fig. 8. Elution of *Y. intermedia* grown at 25°C from DEAE-Trisacryl by a NaCl gradient. The first peak occurs at 0.11 M NaCl.

Discussion

The interaction of bacteria with epithelial cells *in vitro* places the bacteria in four possible locations with respect to the cells: (i) free and unassociated; (ii) attached to inanimate surfaces presented by the procedure; (iii) attached to the epithelial cell

surface; and (iv) inside the epithelial cells. A complete description of bacterial-epithelial cell interactions should include enumeration of the bacteria in the last three locations after efficient removal or destruction of unassociated bacteria.

Measurement of bacterial attachment to inanimate surfaces present in the test system, which has

frequently been ignored, provides a basis for comparing the relative specificity and affinity of the bacteria for the epithelial cells. For example, *Y. enterocolitica* grown at 35°C showed a high degree of attachment for epithelial cells but these bacteria also attached avidly to polystyrene (table V). This changes the interpretation of the relative affinity and adhesion of *Y. enterocolitica* grown at 35°C and 25°C for the epithelial cells.

The correlation between attachment to polystyrene and epithelial cells suggests that both are based on a bacterial surface property that does not involve a specific adhesin-receptor mechanism. A relationship between bacterial surface properties and adhesion to inanimate surfaces has been demonstrated (Paul, 1984). The attachment of bacteria to epithelial cells, at least by non-fimbriate bacteria such as *Y. enterocolitica*, may involve mechanisms that are not different from those involved in attachment of bacteria or proteins to inanimate surfaces such as polystyrene. Cantarero *et al.* (1980) observed that the adsorption of proteins to polystyrene was a characteristic of the particular protein which was not explicable by charge differences, and that binding increased proportionately with incubation time. This is analogous to our observations with *Y. enterocolitica* and epithelial cell monolayers. The linear relationship reported by Cantarero *et al.* (1980) in a log-log plot of bound versus added protein is the same as we observed in a log-log plot of total associated and attached bacteria and the number of bacteria added per monolayer ($r=0.999$).

Enumeration of all bacteria associated with epithelial cells does not provide information about the number of intracellular (i.e., invasive) bacteria, which may or may not accompany attachment. Evidence has been presented that invasion by *Y. enterocolitica* can occur without attachment (Vesikari *et al.*, 1981; Heeseman *et al.*, 1984). We found that increased attachment with *Y. enterocolitica* grown at 35°C was not accompanied by increased invasion (table V). It was expected that, in our assay system, any decrease in attachment or affinity for the epithelial cells would be accompanied by a decrease in the number of intracellular bacteria.

Most reports indicate that growth of *Y. enterocolitica* at 35–37°C results in decreased association with epithelial cells *in vitro*. Lee *et al.* (1977) reported that invasion of HeLa cells did not occur with *Y. enterocolitica* grown at 35°C and Okamoto *et al.* (1980) observed that this growth temperature eliminated attachment to epithelial cells. Martinez (1983) and Schiemann and Swanz (1985b) showed that *Y. enterocolitica* grown at 25°C adhered better

to Henle cells than bacteria grown at 35°C. We found in this study that *Y. enterocolitica* grown at 35°C adhered strongly to polystyrene and Henle 407 epithelial cell monolayers. In other methods, the bacteria grown at 35°C may attach to plastic or other surfaces before they come in contact with the epithelial cells. It is obvious that the method used for observing bacterial-epithelial cell interactions influences the interpretation of the role of growth temperature.

Treatment of epithelial cells with cytochalasin B, which prevents the actin polymerisation necessary for phagocytosis (Axline and Reaven, 1974; Lin *et al.*, 1980), inhibited invasion of Henle 407 epithelial cells but not attachment by *Y. enterocolitica*. This type of inhibition has been reported for other enteroinvasive bacteria (Hale *et al.*, 1979; Bukholm, 1984; Heesemann and Laufs, 1985). A similar inhibition was observed with iodoacetate and 2,4-dinitrophenol, inhibitors of energy metabolism, and with valinomycin, an ionophore that reduces ATP production (unpublished data). Bacterial invasion of epithelial cells appears to resemble receptor-mediated endocytosis (Goldstein *et al.*, 1979) in which uptake is induced by some bacterial structure, as recently suggested for *Shigella* and enteroinvasive *E. coli* (Hale *et al.*, 1985).

It has been suggested that the $(42-48) \times 10^6$ -mol. wt virulence plasmid in *Y. enterocolitica* controls epithelial cell attachment but not invasion. Portnoy *et al.* (1981) concluded that invasion of HEp-2 cells was not plasmid-mediated, a later observation by Schiemann and Devenish (1982) for HeLa cells. Vesikari *et al.* (1981 and 1983) reported that plasmid-positive *Y. enterocolitica* serotypes O:3 and O:9 grown at 37°C were adherent but not invasive for HEp-2 cells, whereas plasmid-negative cultures were invasive but not adherent. Using centrifugation to promote HEp-2 cell association, Vesikari *et al.* (1982) found that plasmid-negative *Y. enterocolitica* O:3 was highly invasive. Heeseman *et al.* (1984) developed a transconjugant of a strain of serotype O:5 that demonstrated adherence but not invasion with HEp-2 cells. We found that a plasmid-negative isogenic culture of *Y. enterocolitica* serotype O:5,27 showed a 70% decrease in total association and an 86% decrease in attachment and invasion (table IV). It appears, therefore, that the virulence plasmid in *Y. enterocolitica* controls some property that affects only the degree of attachment.

Partitioning in a dextran-PEG biphasic system has been used to describe the hydrophobic properties of bacteria. Concentration in the dextran-rich bottom phase is considered to be evidence of hydrophobicity (Edebo *et al.*, 1983). Perers *et al.*

(1977) observed a correlation between hydrophobicity by this method and association of *E. coli* and *Salmonella typhimurium* with mouse intestinal mucosa. Kihlstrom and Magnusson (1983) did not observe a clear distinction between partitioning in dextran-PEG and HeLa cell association among strains of *Y. enterocolitica* serotype O:3. We found that *Y. enterocolitica* serotype O:5,27 grown at 35°C was more hydrophobic than bacteria grown at 25°C which agreed with the relative attachment to both polystyrene and epithelial cell monolayers. This relationship was not observed with *Y. kristenseni* which was the most hydrophobic culture among those examined but showed negligible attachment to both polystyrene and monolayers (table V). These results agree with our previous report that hydrophobicity alone does not explain the association of *Y. enterocolitica* with epithelial cells *in vitro* (Schiemann and Swanz, 1985a).

Interactions with ion exchangers, known as electrostatic interaction chromatography (ESIC) (Hermansson *et al.*, 1982) or ion-exchange chromatography (EIC) (Edebo *et al.*, 1983), has been used to measure electrostatic properties of bacteria. A higher degree of adsorption to an anion exchanger, or elution by a higher salt concentration, is considered to be evidence of a greater tendency toward an electrostatic interaction. Hermansson *et al.* (1982) observed that fimbriate bacteria interacted with DEAE-Sephacel to a greater degree than did nonfimbriate bacteria, which was regarded as evidence of a higher negative charge conferred by the fimbriae. Edebo *et al.* (1983) found that rough *S. typhimurium* eluted from DEAE-Sephacel at a higher salt concentration and also demonstrated greater attachment to animal cells. Kihlstrom and Magnusson (1983) used a similar procedure with *Yersinia* spp. but could not observe any correlation with HeLa cell association. We found that the only large difference between epithelial cell association-positive *Y. enterocolitica* and epithelial cell association-negative *Yersinia* was with Sepharose at pH 3.9. The lower pH could affect both surface charge and hydrophobicity of the bacteria, DEAE and the Sepharose. According to Wood (1980), bacteria adhere to anion exchangers at pH values above their isoelectric point but nonspecific adherence occurs at a pH close to their isoelectric point. The cross-linked Sepharose gel, which is stable at pH 3–14, is more hydrophobic at low pH. The

greater adherence observed at low pH probably results from an increase in hydrophobicity. Although this distinction between epithelial cell association-positive and -negative *Yersinia* is interesting, the conditions under which it occurs are physiologically abnormal and, therefore, the observation is not highly relevant to epithelial cell association and pathogenesis in general.

Elution of isogenic cultures of *Y. enterocolitica* from DEAE-Trisacryl with a continuous NaCl gradient produced two peaks at approximately the same salt concentrations. Cloning the second peak resulted in an identical elution pattern indicating that the original culture was not mixed. Bacteria from both peaks showed similar association with Henle 407 epithelial cells indicating that different degrees of affinity for the anion exchanger did not parallel epithelial cell association. *Y. enterocolitica* grown at 35°C did not show distinct elution peaks, probably because of nonspecific interaction with the gel resulting from the increase in hydrophobicity at this growth temperature. The majority of the bacteria in cultures of *Y. kristenseni* and *Y. intermedia* eluted from DEAE-Trisacryl in one peak at a slightly lower NaCl concentration than associated with the first peak for *Y. enterocolitica*. This agrees with the slightly reduced affinity observed with these cultures on DEAE-Sephacel. There was, therefore, a correlation between epithelial cell association and a small difference in electrostatic interactions.

Enteropathogenic bacteria in general appear consistently to demonstrate physicochemical surface properties that promote adhesion. These characteristics, however, are not always restricted to pathogenic strains. There is also no evidence that the adhesion of enteropathogenic bacteria to epithelial cells involves unique or specific bonding mechanisms. In the multifactorial process of epithelial cell adhesion-invasion, physicochemical surface properties of the bacteria probably present no more than ancillary factors that promote closer apposition of the bacteria with the epithelial cells. Invasion of the epithelial cells is probably more specific in that it is an endocytic process induced by some structure on the bacteria.

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