Invasion of Vero cells by *Salmonella* species

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Summary. The invasiveness of *Salmonella* strains for Vero cells was studied by quantitative bacteriology; the technique was more sensitive than phase contrast microscopy. All of 59 *Salmonella* strains, of 19 different serotypes, were more invasive than *Escherichia coli* K12. Three strains of *Shigella* were as invasive as most of the *Salmonella* strains whereas 29 strains of *E. coli*, two of *Proteus*, three of *Klebsiella* and one of *Serratia* were much less invasive. Two *Citrobacter* strains exhibited intermediate invasiveness. Eleven *Salmonella* strains were also shown to be invasive in HeLa, int 407, bovine kidney, chick kidney and chick embryonic fibroblast cells. The difference between invasive and non-invasive organisms was apparent irrespective of the numbers of bacteria in contact with Vero cells or the duration of bacteria-cell contact. There was little intracellular multiplication of *S. typhimurium* in Vero cells. Unlike the situation with *Shigella*, incubation of *Salmonella* or *Salmonella*-cell mixtures at 41°C, 22°C or 0°C had little effect on invasiveness. Non-viable *Salmonella* organisms were non-invasive. Incubation of Vero cells with cholera toxin, dinitrophenol, iodoacetic acid, cytochalasin B or D-mannose did not substantially reduce invasiveness. Virulence-associated plasmids were not essential to invasion by *S. typhimurium*, *S. gallinarum* or *S. pullorum*. Neither somatic antigens nor mannose-sensitive haemagglutinins were essential to the invasiveness of an *S. infantis* strain, but an additional factor, eliminated by *N*-methyl, *N*-nitro, *N*-nitrosoguanidine mutagenesis did contribute to invasiveness.

Introduction

Salmonella spp. are normally transmitted by the faecal-oral route (Buxton, 1957) and the importance of invasion via the alimentary tract to the virulence of serotypes that characteristically produce systemic disease, such as *S. typhi*, *S. dublin*, *S. choleraesuis*, *S. gallinarum* and *S. abortus-equi*, is self evident. In some cases invasiveness has been demonstrated experimentally (Smith, 1955; Smith and Jones, 1967; Smith and Halls, 1968; Tannock *et al.*, 1975). Invasiveness of *S. typhimurium*, which typically produces severe systemic disease in young animals, has also been demonstrated in newly-hatched chickens (Barrow *et al.*, 1987a) and in calves (Clarke and Gyles, 1985). Similarly, the passage of *S. enteritidis* and *S. thompson* through and between intestinal mucosal cells, as the first stage of systemic infection of newly-hatched chickens, has been demonstrated by electron microscopy (Turnbull and Richmond, 1978; Popiel and Turnbull, 1985).

Most other serotypes are usually associated only with human gastroenteritis (food poisoning). Mu-

cosal invasion can be demonstrated microscopically in natural and experimental cases of gastroenteritis produced by *S. typhimurium* in man (Day *et al.*, 1978) and rhesus monkeys (Kent *et al.*, 1966; Giannella, 1975). The extent to which invasiveness is involved in colonisation of the alimentary tract in animals in the absence of disease is unknown but invasive serotypes may be shed in chicken faeces for shorter periods than non-invasive strains (Barrow *et al.*, 1988).

Understanding of the basis of invasiveness of another enteric pathogenic genus, *Shigella*, has increased considerably by use of tissue culture to study invasion *in vitro* (Sansone *et al.*, 1982, 1983, 1986; Hale *et al.*, 1983; Watanabe and Timmis, 1984; Watanabe and Nakamura, 1986). In contrast, knowledge of the interactions between *Salmonella* spp. and non-phagocytic cells is fragmentary. Invasion of HeLa and HEp-2 cells by *S. typhimurium* has been demonstrated *in vitro* (Giannella *et al.*, 1973; Kihlstrom and Edebo, 1976; Kihlstrom and Latkovic, 1978; Jones *et al.*, 1981; Small *et al.*, 1987). However, little is known of the microbial determinants and cell receptors involved, or how these relate to host specificity.

Irreversible attachment to HeLa cells is a prerequisite to invasion by *S. typhimurium* (Jones *et al.*, 1981). Production of a mannose-resistant haem-agglutinating adhesin, involved in the pre-invasion attachment stage, was thought to be reduced by culture at low temperatures (Jones and Richardson, 1981) as occurs with *Shigella* spp. (Maurelli *et al.*, 1984), but later work has not confirmed this (Small *et al.*, 1987). Jones *et al.* (1982) suggested that adhesion to, and invasion of, HeLa cells was mediated by the 85-kb virulence-associated plasmid found in this serotype. However, this finding also has not been confirmed (Pardon *et al.*, 1986; Gulig and Curtiss, 1987). Kihlstrom and Edebo (1976) found that "rough" cell wall mutants were more invasive, since these showed increased hydrophobicity and attached to cells in greater numbers. Unlike *Sh. flexneri*, *S. typhimurium* does not multiply rapidly intracellularly (Devenish and Schiemann, 1981; Sansonetti *et al.*, 1986; Small *et al.*, 1987).

The above studies have generally been limited to *S. typhimurium*. We have compared invasion of several different cell types by various *Salmonella* spp. and have selected one for further study of the kinetics of invasion and of the microbial determinants that might be involved.

Materials and methods

Bacterial strains

Strains of *S. typhimurium*, *S. anatum*, *S. enteritidis*, *S. giv*, *S. hadar*, *S. heidelberg*, *S. indiana*, *S. infantis*, *S. kedougou*, *S. kentucky*, *S. ohio*, *S. oranienburg*, *S. presov* and *S. virchow* were isolated from cases of human food poisoning or from the alimentary tract of healthy chickens. Additional strains of *S. typhimurium* and strains of *S. gallinarum*, *S. pullorum*, *S. dublin*, *S. cholerae-suis*, *S. abortus-ovis* and *S. abortus-equi* were isolated from cases of systemic animal salmonellosis. *Shigella* strains were originally isolated from cases of human dysentery. *Escherichia coli* strains obtained from the culture collection at this laboratory were originally isolated from healthy pigs and calves and cases of porcine bowel oedema, calf and chicken septicaemia, bovine mastitis and diarrhoea in pigs, calves and man. *Citrobacter*, *Klebsiella*, *Proteus* and *Serratia* strains were isolated from the alimentary tract of various species.

All bacterial strains were maintained on Dorset's egg slopes at 4°C. Broth cultures were made in 10-ml volumes of Nutrient Broth (Oxoid, CM67), incubated at 37°C for 24 h in a water bath with shaking. Such cultures usually contained between 8×10^8 and 2×10^9 cfu/ml.

Full details of the production of mutants of *S. typhimurium* strain F98, *S. infantis* strain 1326/28, *S. gallinarum* strain 9 and *S. pullorum* strain 3 were described by Barrow *et al.* (1988). Non-flagellate mutants of the *S.*

typhimurium and *S. infantis* strains and a mannose-sensitive non-haemagglutinating mutant of the *S. infantis* strain were obtained by *N*-methyl, *N*-nitro, *N*-nitroso-guanidine (NTG) mutagenesis as described by Barrow *et al.* (1988).

"Rough" cell-wall mutants of *S. typhimurium*, *S. gallinarum* and *S. pullorum* were prepared as follows: bacteriophages, lytic for each strain, were isolated from sewage (Barrow, 1986). Plates of Tryptose Agar (Difco) were flooded with equal volumes of broth cultures of the bacteria and a dilution of phage which would just produce confluent lysis. Rough mutants were selected by their granular consistency or by one of the following characteristics: non-agglutination with appropriate O-antiserum (Wellcome Diagnostics), spontaneous agglutination in physiological saline, or agglutination by acriflavine 0.2% (Smith, 1965).

The 85-kb virulence plasmids of the *S. gallinarum* and *S. pullorum* strains were cured by methods described previously (Barrow *et al.*, 1987b; Barrow and Lovell, 1988). The plasmid carrying transposon 3 (pSC101::Tn3), which was used to "tag" the virulence plasmid of *S. typhimurium* F98, was introduced by mobilisation with the I plasmid in a prototrophic strain of *E. coli* K12. The tagged virulence plasmid was eliminated by incubation at 42°C for 18 h as used for *S. gallinarum* 9 (Barrow *et al.*, 1987b).

Cell culture

All cells were grown as monolayers at 37°C in a CO₂ incubator. Vero, HeLa, human intestine 407 and bovine kidney cell lines were obtained from Flow Laboratories. Vero cells were grown in 199 medium (Flow) containing fetal calf serum 10% and L-glutamine 1%. HeLa and bovine kidney cells were cultured in Eagle's minimal essential medium containing fetal calf serum 10%, glutamine 1% and non-essential amino acids 1%. Intestine 407 cells were cultured in Eagle's medium containing Hanks's Balanced Salts Solution (HBSS), calf serum 15% and glutamine 1%.

Primary chicken kidney cells were prepared from the kidneys of 2–3 weeks old chickens. Kidneys were removed aseptically, teased and trypsinised with Versene (NaCl 0.8%, KH₂PO₄ 0.02%, KCl 0.02%, Na₂HPO₄ 0.15%, EDTA 0.02%). The cells obtained were cultured in 199 medium containing Tryptose Phosphate Broth (Difco) 10%, NaHCO₃ 0.8%, calf serum 8%, amphotericin B 0.04% and gentamicin sulphate 0.001%.

Primary chicken embryo fibroblasts were prepared from 10-day-old embryonated eggs. The embryo was decapitated, eviscerated and macerated. The macerate was washed and trypsinised. Cells thus obtained were cultured in a medium similar to that used for chicken kidney cells, but without amphotericin B or gentamicin.

Invasion of cells

The method was similar to that of Peerbooms *et al.* (1984, 1985). Briefly, bacterial cultures were diluted in

modified HBSS. For most organisms a 1 in 200 dilution was used. However, for strains such as *S. gallinarum*, *S. pullorum*, *S. abortus-equi* and *S. abortus-ovis* which did not grow well in broth, and for cultures grown at lower temperatures, the dilutions were reduced so that the inoculum was similar to that of the control. Monolayers of cultured cells, in 24-well "Linbro" plates (Flow Laboratories), were washed twice in HBSS and were then incubated with the bacterial suspension for 2 h at 37°C in air. At this point the number of viable bacteria/ml of suspension in contact with the cells was counted. The monolayer was washed once in HBSS and subsequently incubated for 1.5 h at 37°C in 199 medium containing kanamycin 250 µg/ml to kill extracellular bacteria (Okamura *et al.*, 1983). The cells were then washed twice with HBSS and lysed for 30 min at 37°C with trypsin 0.025% and Tween 20 1% in 0.01 M NaH₂PO₄ at pH 8. Intracellular and extracellular bacteria were counted on nutrient-agar plates.

In all experiments, invasive (*S. typhimurium* F98) and non-invasive (*E. coli* K12, prototrophic) control strains were included.

For phase contrast microscopy fragments of sterile glass coverslips were laid on the base of the wells before cell culture. Suspensions of bacteria were incubated with the cells in the usual way but immediately before lysis the coverslips were removed for observation.

Effect of cytochalasin B, metabolic inhibitors and cholera toxin

Cytochalasin B (Sigma), dissolved in dimethyl sulphoxide, and two metabolic inhibitors, 2, 4-dinitrophenol and iodoacetic acid (Sigma), were incorporated in the modified HBSS medium at the desired concentrations for the 2-h period of incubation with bacteria. Cholera toxin

(Sigma), diluted in modified HBSS, was incubated with Vero cells for 3 h before and for the 2-h duration of incubation with bacteria.

Sensitivity to kanamycin

Selected strains were tested for their sensitivity to kanamycin. Doubling dilutions of kanamycin in the range 500–1.0 µg/ml were prepared in Sensitivity Test Agar (Oxoid CM409). Plates were inoculated with 0.02 ml of a dilution of each strain, containing *c.* 10⁴ cfu/ml. Minimum inhibitory concentration (MIC) values were calculated after overnight incubation at 37°C.

Results

Validation of the method

The invasiveness of selected organisms for Vero cells, as assessed by quantitative bacteriology and by phase-contrast microscopy, is shown in table I. The quantitative bacteriological invasion test was replicated five times for *S. infantis* and ten or more times for the other strains. *S. typhimurium*, *S. infantis* and *Sh. flexneri* were the most invasive, the numbers of organisms/ml of lysate being between 0.1% and 1% of the inoculum of *c.* 10⁷ cfu/ml. *S. gallinarum* and *S. pullorum* exhibited intermediate invasiveness and the *E. coli* K12 strain was virtually non-invasive. For all six strains the standard errors were small, suggesting that the method was reproducible. Because of this, subsequent results are presented as the mean values from two separate wells examined simultaneously.

Table I. Invasiveness of *Salmonella* spp. *Sh. flexneri* and *E. coli* K12 for Vero cells

Bacterial strain (n)*	Log ₁₀ cfu/ml and standard error (range) in		Invasiveness by phase contrast microscopy
	suspension added to Vero cells	Vero-cell lysate	
<i>S. typhimurium</i> F98 (17)	7.3 ± 0.4 (6.8–7.7)	4.9 ± 0.4 (4.6–5.2)	+
<i>S. infantis</i> 1326/28 (5)	7.6 ± 0.2 (7.3–7.9)	4.6 ± 0.2 (4.4–4.9)	+
<i>Sh. flexneri</i> 59 (11)	6.9 ± 0.1 (6.8–7.1)	4.2 ± 0.2 (3.8–4.6)	+
<i>S. gallinarum</i> 9 (11)	6.9 ± 0.1 (6.8–6.9)	3.3 ± 0.1 (3.0–3.6)	–
<i>S. pullorum</i> 3 (10)	7.0 ± 0.1 (6.9–7.1)	2.5 ± 0.1 (2.3–2.9)	–
<i>E. coli</i> K12 (17)	7.0 ± 0.2 (6.7–7.3)	0.8 ± 0.5 (< 1.0–1.8)	–

* n = Number of times test was replicated.

Ten selected *Salmonella* strains, *Sh. flexneri* and *E. coli* K12 were retested by quantitative bacteriology for invasiveness in HeLa, human intestine 407, bovine kidney, chicken kidney and chick embryo fibroblast cells. Results were very similar to those described for Vero cells; *S. gallinarum* and *S. pullorum* were no more invasive in chicken cell types than they were in cells of mammalian origin. Because of these results all further work was carried out on Vero cells only.

To show that differences in invasiveness were not the results of differences in susceptibility to kanamycin, MICs of kanamycin for selected invasive and non-invasive strains were calculated. MICs of kanamycin for invasive *Salmonella* strains, *Sh. flexneri* 59, *S. gallinarum* 9, *S. pullorum* 3, one *Citrobacter* strain, five "wild" strains of *E. coli* and *E. coli* K12 were in the range $\leq 1-4 \mu\text{g/ml}$. The MIC of kanamycin for *E. coli* K12 carrying plasmid pML21, encoding kanamycin resistance, was $> 500 \mu\text{g/ml}$.

Viable counts obtained from Vero-cell lysates, estimated after carrying out an invasion test with the kanamycin-resistant and -sensitive strains of *E. coli* K12, *S. typhimurium* F98 and *S. gallinarum*, were ($\log_{10}\text{cfu}$) 6.4, 1.8, 4.2 and 3.1 respectively.

By phase contrast microscopy $\leq 1\%$ of Vero cells appeared to be infected by *S. typhimurium*, *S. infantis* or *Sh. flexneri*. Invasive bacteria, sometimes several together, were present in intracellular vacuoles; the *Salmonella* strains were easily distinguishable by their motility. In the case of *S. gallinarum*, *S. pullorum* and *E. coli* K12 no intracellular organisms were visible.

Similarly, cells of *S. typhimurium* F98 that had been killed by formaldehyde, ethanol, chloroform or by heating at 58°C for 30 min appeared non-invasive.

Invasiveness of Salmonella and other genera

The invasiveness of 20 *Salmonella* serotypes and of other members of the Enterobacteriaceae for Vero cells is shown in table II. Of the genera tested *Salmonella* was the most invasive. The highest viable count obtained from the Vero-cell lysate, ($10^{6.1}\text{cfu}$), was obtained with one strain of *S. typhimurium*, and many strains of *S. typhimurium*, *S. dublin*, *S. cholerae-suis* and various food-poisoning serotypes produced lysate counts of $> 10^5 \text{cfu}$. *S. gallinarum*, *S. pullorum*, *S. abortus-equi* and *S. abortus-ovis* exhibited intermediate invasiveness.

Of the other genera tested, *Shigella* was most invasive. Several strains of *E. coli* and two *Citrobacter* strains were more invasive than *E. coli* K12.

Table II. Invasiveness of *Salmonella* and other genera for Vero cells

Species tested (n)	$\log_{10} \text{cfu/ml}$ of Vero-cell lysate
<i>S. typhimurium</i> (29)	5.3 (4.5-6.1)*
<i>S. gallinarum</i> (2)	2.6, 3.3
<i>S. pullorum</i> (1)	2.5
<i>S. dublin</i> (3)	5.4, 5.5, 6.0
<i>S. cholerae-suis</i> (3)	4.3, 5.3, 5.4
<i>S. abortus-ovis</i> (1)	3.2
<i>S. abortus-equi</i> (1)	2.5
<i>S. enteritidis</i> (3)	4.3, 4.7, 4.8
<i>Salmonella</i> spp. (17) (12 serotypes)†	5.1 (4.5-5.4)
<i>Sh. flexneri</i> (1)	4.2
<i>Sh. sonnei</i> (1)	3.5
<i>Sh. dysenteriae</i> (1)	3.6
<i>E. coli</i> K12 (1)	0.8
<i>E. coli</i> (27)‡	1.9 (<1.0-2.9)
<i>E. coli</i> (2)¶	1.9, 2.6
<i>Citrobacter</i> spp. (2)	3.3, 2.9
<i>Proteus</i> spp. (2)	2.3, 2.0
<i>Klebsiella</i> spp. (3)	1.0, 1.6, 1.9
<i>S. marcescens</i> (1)	1.0

(n) = Number of strains tested.

*Median count with range in parentheses; for 1-3 strains, all values are given.

†Food-poisoning serotypes *S. anatum* (2), *S. givé* (1), *S. hadar* (4), *S. heidelberg* (1), *S. indiana* (1), *S. infantis* (1), *S. kedougou* (1), *S. kentucky* (1), *S. ohio* (1), *S. oranienburg* (1), *S. presov* (1), *S. virchow* (2).

‡Nine strains from bovine mastitis, 8 enteropathogenic strains from pigs, calves and man, 2 strains from porcine bowel oedema, 3 strains from calf septicaemia and 3 strains from chicken septicaemia.

¶Two non-pathogenic porcine strains.

There was no association between the number of *E. coli* recovered from the Vero-cell lysate and the disease or the host of origin. The *Proteus*, *Klebsiella* and *Serratia* strains examined displayed little or no invasiveness.

Kinetics of Vero-cell invasion

The effect of bacterial numbers on invasion. In experiments in which dilutions of cultures of *S. typhimurium* F98, *S. gallinarum* 9 and *E. coli* K12 were incubated with Vero cells, the numbers of bacteria recovered from the Vero cell lysate increased with the inoculum (fig. 1). The differences in invasiveness of the three strains were apparent throughout the range of dilutions. Invasion by the *S. typhimurium* strain was detectable with an inoculum of $c. 10^3 \text{cfu/ml}$. No further increase in recovery from the cell lysate was observed with any of the strains when the bacterial inoculum was raised from 10^8 to $c. 5 \times 10^8 \text{cfu/ml}$.

Fig. 1. Recovery of bacteria from Vero-cell lysate after addition of different inocula. Each point represents arithmetic mean of two values. ▲ *S. typhimurium* F98; ■ *S. gallinarum* 9; ● *E. coli* K12.

The effect of contact time. The recovery of *S. typhimurium* F98 and *E. coli* K12 from Vero-cell lysates after contact for varying times is shown in fig. 2. The number of organisms per ml of lysate increased with time, but after 60 min the rate of increase was small. The number of organisms in contact with the cells remained constant during the course of the experiment. The viable count of *S. typhimurium* in the lysate was always much greater than that of *E. coli* K12.

Intracellular multiplication of *S. typhimurium* and *E. coli* K12. After incubation in the presence of

Fig. 2. Recovery of bacteria from Vero-cell lysate as a function of incubation time. Each point represents arithmetic mean of two values. ▲ Number of *S. typhimurium* F98 in contact with Vero cells; ● number of *E. coli* K12 in contact with Vero cells; △ number of *S. typhimurium* F98 in cell lysate; ○ number of *E. coli* K12 in cell lysate.

kanamycin, the number of *S. typhimurium* F98 recovered from cell lysate gradually increased fourfold from $10^{3.9}$ to $10^{4.5}$ cfu at 6 h. On further incubation, the number recovered fell, reaching 10^4 cfu at 24 h. At this time there was evidence of kanamycin toxicity to the cells, which had started to round up and become detached. With *E. coli* K12 the number of bacteria recovered was very low or undetectable throughout the experiment.

The effect of temperature. The ability of *S. typhimurium*, *S. hadar*, *S. virchow*, *S. heidelberg* and *Sh. flexneri*, cultured at 37°C or 22°C, to invade Vero cells held at 37°C, 22°C or 0°C during the invasion period was assessed. The final lysis step was carried out at 37°C as usual. The results are shown in table III. When bacterial culture and the invasion test were performed at 37°C or 22°C, all strains were fully invasive. When bacteria cultured at 37°C were tested for invasiveness at 0°C by holding the tissue culture plate on ice in a cold room at 4°C, the invasiveness of all strains was reduced over 100-fold. Nevertheless $>10^3$ cfu of salmonellae were recovered per ml of cell lysate. *Salmonella* strains cultured at 22°C were all fully invasive when tested at 37°C or 22°C. In contrast, the invasiveness of *Sh. flexneri* cultured at 22°C was reduced over 1000-fold at 37°C and almost 100-fold at 22°C.

Single strains of *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. cholerae-suis* and *E. coli* K12 cultured at 41°C were tested in Vero cells held at 41°C to investigate invasiveness at the blood temperature of the chicken. All strains were equally invasive at 41°C and 37°C.

The effect of carbohydrates

The inclusion of D(+)mannose 1% in the bacterial suspension medium had no effect on the invasiveness of eleven strains of *Salmonella* (10 serotypes). Similarly D(-)arabinose, D(+) raffinose, N-acetyl-D-glucosamine, D(+) galactose and α-D(+)fucose (all 1%) had no effect on the invasion of two strains of *S. typhimurium*.

The effect of metabolic and phagocytic inhibitors

The effects of incubating cell-bacteria mixtures with inhibitors of metabolism (2,4-dinitrophenol and iodoacetic acid) and of phagocytosis (cytochalasin B and cholera toxin) are shown in table IV. Dinitrophenol reduced the number of bacteria recovered from the cell lysate by up to 96% (*S. typhimurium*) and 98% (*Sh. flexneri*) of the chemical-free control counts. However, the number of viable bacteria in contact with the cells also declined.

Table III. The effect of temperature on Vero cell invasion

Bacterial strain	Log ₁₀ viable count/ml of Vero-cell lysate when bacteria or bacteria + Vero cell mixture were incubated at the following temperatures				
	Bacteria cultured at 37°C			Bacteria cultured at 22°C	
	Bacteria + Vero cells held at			Bacteria + Vero cells held at	
	37°C	22°C	0°C	37°C	22°C
<i>S. typhimurium</i> F98	5.4	5.5	3.2	4.1	4.4
<i>S. hadar</i> 1481/79	5.4	5.4	3.4	4.7	4.2
<i>S. virchow</i> 961	5.8	5.8	3.6	4.7	4.9
<i>S. heidelberg</i> 17705	5.8	6.1	3.5	3.7	4.6
<i>Sh. flexneri</i> 59	3.8	3.3	1.8	0.7	1.5

Table IV. The effect of metabolic and phagocytic inhibitors on Vero cell invasion

Organism	Chemical	Log ₁₀ cfu/ml	
		in contact with Vero cells	in Vero cell lysate
<i>S. typhimurium</i>	None	7.3	5.1
	dinitrophenol 0.01 mM	7.0 (−55)*	4.9 (−34)*
	dinitrophenol 0.1 mM	7.1 (−34)	4.9 (−33)
	dinitrophenol 1 mM	6.6 (−80)	4.5 (−75)
	dinitrophenol 2 mM	6.5 (−85)	3.8 (−96)
<i>S. typhimurium</i>	None	7.4	4.9
	iodoacetic acid 0.01 mM	7.4 (+3)	4.7 (−30)
	iodoacetic acid 0.1 mM	7.2 (−35)	4.4 (−66)
	iodoacetic acid 0.2 mM	7.3 (−31)	4.0 (−86)
	cytochalasin B 0.1 µg/ml	7.4 (−9)	4.4 (−63)
	cytochalasin B 1 µg/ml	7.5 (+9)	4.7 (−38)
	cytochalasin B 2 µg/ml	7.6 (+32)	4.2 (−79)
	cytochalasin B 4 µg/ml	7.2 (−35)	4.6 (−49)
	cytochalasin B 8 µg/ml	7.6 (+27)	4.4 (−68)
<i>S. typhimurium</i>	None	6.8	3.8
	cholera toxin 1 µg/ml	7.2 (+62)	4.6 (+85)
	cholera toxin 10 µg/ml	7.1 (+51)	4.6 (+85)
	cholera toxin 100 µg/ml	7.2 (+65)	3.9 (+13)
<i>Sh. flexneri</i>	None	6.9	2.9
	dinitrophenol 2 mM	5.5 (−95)	1.5 (−98)
	iodoacetic acid 0.2 mM	6.2 (−80)	0.7 (−99.5)
	cytochalasin B 8 µg/ml	6.6 (−44)	0.7 (−99.5)
	None	6.8	3.1
	cholera toxin 100 µg/ml	6.8 (−11)	2.6 (−71)

*Percentage change in viable count compared to chemical free control.

Cytochalasin B and iodoacetic acid produced smaller and variable reductions in invasion by *S. typhimurium*, but invasion of *Sh. flexneri* was reduced by >99%. Cholera toxin apparently caused small increases in the counts of *S. typhimurium* in contact with the cells as well as in the cell lysate; invasiveness of *Sh. flexneri* was slightly reduced.

The number of *S. typhimurium* F98 recovered from the cell lysate always exceeded 10^{3.8} cfu.

Vero-cell invasion by *Salmonella* mutants

The results of assessing the invasiveness of mutants of *S. gallinarum* 9, *S. pullorum* 3, *S.*

typhimurium F98 and *S. infantis* 1326/28 are shown in table V. For this experiment, the inoculum of *S. pullorum* was increased by a factor of 10. Mutants of *S. gallinarum* and *S. pullorum* which had been cured of their virulence-associated plasmids were as invasive as the parent strain. A fivefold reduction was observed in the invasiveness of a variant of *S. typhimurium* cured of a virulence plasmid. The rough cell-wall mutants of *S. gallinarum*, *S. typhimurium* and *S. infantis* and a mutant of *S. infantis* which did not produce mannose-sensitive haemagglutinins (MSHA) were as invasive as the parent strains. Smooth non-motile mutants of *S. typhimurium* and *S. infantis* were examined. The *S. infantis* also possessed MSHA. The invasiveness of the *S. typhimurium* mutant was reduced 20-fold and that of the *S. infantis* mutant 200-fold. An additional mutant of *S. infantis* which was smooth, motile and possessed MSHA was created by NTG treatment. The invasiveness of this mutant was reduced almost 100-fold.

Discussion

These results show that bacterial invasiveness can be quantified readily *in vitro* in Vero, HeLa, bovine and chicken kidney, chick embryo fibroblast and human intestinal cell monolayers. This extends previous findings with HeLa (Giannella *et al.*, 1973; Kihlstrom and Edebo, 1976; Kihlstrom and Latkovic, 1978; Jones *et al.*, 1981) and HEp-2 cells (Small *et al.*, 1987).

Quantitative bacteriology with Vero cells was reproducible and was able to detect intermediate invasiveness in bacteria that appeared non-invasive

by microscopy. Intermediate invasiveness is unlikely to be due to incomplete killing of extracellular bacteria since there was little difference in the kanamycin sensitivity of strains that exhibited varying abilities to invade.

Salmonella and *Shigella* were the most invasive genera. Some of the serotypes which produce systemic disease in a limited number of host species, such as *S. gallinarum*, *S. pullorum*, *S. abortus-ovis* and *S. abortus-equi*, appeared less invasive than others. The reason for this is unclear. Some strains of *E. coli* and *Citrobacter* were moderately invasive, but there was no association between the degree of invasiveness of the *E. coli* strains and the disease or host of origin. None of the *E. coli* strains studied were of the enteroinvasive type (Small *et al.*, 1987). *Citrobacter* strains can produce systemic disease in man (Holmes and Gross, 1983) and it would be interesting to assess the rôle of invasiveness in the pathogenicity of this genus.

Invasion by *S. typhimurium* was detectable even when the number present was as low as 10^3 – 10^4 cfu/ml. Invasion was very rapid, but this may reflect rapid, irreversible attachment followed by incomplete killing by kanamycin during the phase of uptake. This suggests that irreversible attachment may occur more quickly than the 5 min suggested by Jones *et al.* (1981). The rate of increase in the numbers of intracellular bacteria reached a maximum after 1–2 h contact time. Since a relatively small percentage of Vero cells contained bacteria, this suggests that monolayers may be heterogenous in terms of susceptibility. The minimal intracellular multiplication of the *S. typhimurium* strain is not surprising since multiplication in mucosal cells is

Table V. Vero cell invasion by *Salmonella* mutants

Strain characteristic	Log ₁₀ viable count/ml of Vero-cell lysate			
	<i>S. gallinarum</i> 9	<i>S. pullorum</i> 3	<i>S. typhimurium</i> F98	<i>S. infantis</i> 1326/28
Parent	3.0	3.2	5.7	4.7
Rough cell-wall*	3.4	NT	5.7	4.5
Non-motile†	...	...	4.4	2.2
Non-haemagglutinating‡	...	...	...	4.3
Virulence plasmid-less	3.2	3.3	5.0	...
NTG-treated‡	NT	NT	NT	2.6

NT = Not tested.

... = Not relevant.

*Induced by bacteriophage.

†Induced by NTG treatment.

‡This mutant was motile and smooth and possessed mannose-sensitive haemagglutinins.

not a characteristic of the pathogenicity of *Salmonella* spp.

Live bacteria were required for invasion. Hale *et al.* (1979) also found that metabolically active cells of *Sh. flexneri* 2a were required to stimulate endocytosis by Henle intestinal epithelial cells. The present finding, that lowering the ambient temperature of the Vero cells to 0°C considerably reduced invasion by *Salmonella* spp. and *Sh. flexneri*, supports the contention that eukaryotic cellular activity contributes considerably to invasion by both genera. At 0°C *Salmonella* spp. were more invasive than *Sh. flexneri*, suggesting that the bacterial contribution to invasion by *Salmonella* spp. may be greater. In support of this, relatively small reductions in *S. typhimurium* invasiveness were induced by cholera toxin and cytochalasin B, chemicals which directly affect cell phagocytosis (Cox and Karnovsky, 1973; Axline and Reven, 1974) and by iodoacetic acid and dinitrophenol, inhibitors of cell metabolism.

A difference between *Shigella* and *Salmonella* was also seen when the bacteria were grown at a reduced temperature. Growth at 22°C profoundly reduced the invasiveness of *Sh. flexneri* 59 as reported previously (Maurelli *et al.*, 1984), while having no effect on the invasiveness of four *Salmonella* strains. This effect has also been reported previously (Small *et al.*, 1987). It is likely, therefore, that the microbial determinants of invasion in these two genera are very different.

The involvement of MSHA (type-1 pili) in invasion by *Salmonella* spp. is unlikely because D-mannose did not reduce the invasion by 11 *Salmonella* strains, and a mutant of *S. infantis* which did not produce MSHA was, nonetheless, invasive.

Rough strains were as invasive as the smooth parent strains. This is in contrast to the findings of Kihlstrom and Edebo (1976) who found that their rough strains were more adherent and thus more invasive. The involvement of flagellar antigens is less clear. Jones *et al.* (1981) suggested that motility facilitates initial contact with cells. Carsiotis *et al.* (1984) also found that the possession of flagella was a virulence characteristic for *S. typhimurium*. In our study, non-motile strains of *S. typhimurium* and *S. infantis* were less invasive, suggesting that motility might indeed contribute to invasion. An additional unidentified factor was present in *S. infantis* which could be eliminated by NTG.

The virulence-associated plasmids of *S. typhimurium*, *S. gallinarum* and *S. pullorum* were not essential to Vero-cell invasion by these strains. This agrees with the in-vivo findings of Pardon *et al.* (1986) and Gulig and Curtiss (1987). However, Barrow *et al.* (1987b), found that the virulence plasmid of *S. gallinarum* contributed to invasion in older chickens but not in newly-hatched birds, perhaps because the complex gut flora of older birds hindered colonisation by the plasmid-cured variant. Phagocytic cells have also been demonstrated engulfing bacteria in the intestinal lumen (Popiel and Turnbull, 1985). It is likely, therefore, that invasion *in vivo* is multifactorial. Use of a cell-culture system *in vitro* should simplify experimental work on invasion by eliminating the need to consider colonisation and by eliminating the involvement of phagocytic cells.

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