The Invasion of HeLa Cells by *Salmonella typhimurium*: Reversible and Irreversible Bacterial Attachment and the Role of Bacterial Motility

By G. W. JONES,* LEIGH A. RICHARDSON AND
DOROTHY UHLMAN

*Department of Microbiology and Immunology, University of Michigan, Ann Arbor,
Michigan 48109, U.S.A.*

(Received 8 December 1980; revised 18 February 1981)

The interactions which brought about the invasion of HeLa cells by *Salmonella typhimurium* consisted of a sequence of three phases. Initially, the motility of the bacteria facilitated their contact with the HeLa cells whereupon the bacteria became attached in a reversible manner (i.e. the bacteria could be removed readily by washing the HeLa cell monolayers with Hanks' Balanced Salt solution). The binding forces responsible for reversible attachment were probably the weak long-range forces of the secondary minimum level of attractive interactions between the bacterium and the HeLa cell. Reversible attachment was a necessary interlude before the bacteria became irreversibly attached to the surfaces of the HeLa cells (i.e. the bacteria were no longer removed by the washing procedure that removed the reversibly attached salmonellae). Irreversible attachment was prevented in solutions of low ionic strength; the forces responsible were probably those of the primary minimum generated between the HeLa cell and a bacterial adhesin which was capable of acting over only short distances between the reversibly attached bacterium and the HeLa cell (i.e. probably less than 15 nm). Only irreversibly attached bacteria proceeded to the third phase and were internalized by the HeLa cells.

INTRODUCTION

The invasion of animal cells by *Salmonella typhimurium* and the active engulfment of bacteria or large particles by professional phagocytes are similar in that both appear to involve endocytic mechanisms. The pertinent aspects of phagocytosis have been reviewed (Stossel, 1975; Silverstein *et al.*, 1977). The entry of salmonellae into animal cells resembles phagocytosis in respect to the morphological responses of the animal cell (Kihlström & Latkovic, 1978) and in that both appear to be driven by energy derived from the animal cell (Stossel, 1975; Kihlström & Nilsson, 1977). Moreover, the internalization of *S. typhimurium* by HeLa cells, like phagocytosis, is inhibited by cytochalasin B (Kihlström & Nilsson, 1977), a drug which is thought to interfere with the function of the microfilaments. The endocytosis of large particles by phagocytes, however, proceeds only if stable bonds of sufficient strength (Capo *et al.*, 1978) are maintained between the particle and the phagocyte throughout the internalization process (Griffin *et al.*, 1975). If the same conditions were fulfilled during the entry of salmonellae into HeLa cells, then the bacterium also must exhibit some form of stable attachment to the host-cell surface prior to and during entry. The attachment of *S. typhimurium* to HeLa cells has been observed (Kihlström & Latkovic, 1978) but was most obvious when rough cell-wall mutants were examined. It was concluded that the increased hydrophobic properties of rough mutants promoted attachment and that bacteria without such cell-wall defects were less adhesive (Kihlström & Edebo, 1976).

The present study of the attachment of wild-type *S. typhimurium* to HeLa cell monolayers revealed that both an unstable reversible form of attachment and a more stable form of irreversible attachment occurred before the bacteria were internalized. Both facets of the attachment of salmonellae to HeLa cells were studied in detail in order to elucidate the nature of these properties and to evaluate the contribution that each made to the internalization process.

METHODS

Salmonella typhimurium strains and the isolation of non-motile mutant salmonellae. Cultures of six wild-type strains were used. Cultures of two FIRM biotypes (Duguid *et al.*, 1975) designated S850 and S2204 were provided by Professor J. P. Duguid (Bacteriology Department, Ninewells Hospital, University of Dundee, U.K.). Dr P. Gemski (Walter Reed Army Institute, Washington, D.C., U.S.A.) supplied cultures of strains TML and W118 which were known to be virulent and to be internalized by HeLa cells (Giannella *et al.*, 1973), and guinea-pig intestinal epithelial cells (Takeuchi, 1967). Strains NY and PR which had been isolated from outbreaks of salmonellosis in two separate areas of the U.S.A. were provided by Dr E. J. Gangarosa (Center for Disease Control, Atlanta, Ga, U.S.A.).

Non-motile mutants of strains S850 and TML were selected from semi-solid agar plates (Freter *et al.*, 1979) seeded with cultures mutagenized with u.v. irradiation or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) as described (Jones & Freter, 1976). Spontaneous motile revertants of these strains were isolated from swarm zones which developed in the same medium.

All cultures were examined for motility by phase-contrast microscopy after the bacteria had been grown in Brain-Heart Infusion broth (BHI; Difco) with shaking for 16 h at 37 °C, and resuspended in Hanks' Balanced Salt solution (Gibco, Grand Island, N.Y., U.S.A.). The proportion of motile bacteria within a prescribed area of the microscope field was recorded. Cultures were examined for rough cell-wall characteristics with phage (Wilkinson *et al.*, 1972). Smooth-specific phages P22 *vir*3, 9NA and KB41 were obtained from Dr B. A. D. Stocker (Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif., U.S.A.). The rough/smooth-specific phage FO and rough-specific phages Br60, Br2, Ffm and 6SR were obtained from Dr P. Gemski. All cultures were smooth and motile unless otherwise stated.

HeLa cell and bacterial cultures. HeLa cells of strain S3 (Microbiological Associates, Bethesda, Md., U.S.A.) were grown as monolayers (about 1.5×10^5 HeLa cells at 50% confluency) on 22 mm cover-slips in Multiplate dishes (Lux Scientific Corp., Newbury Park, Calif., U.S.A.). The medium used was Eagle's Minimal Essential Medium in a Hanks' Balanced Salt Solution base (Gibco) supplemented with 5% (v/v) foetal calf serum (KC Biological, Lenexa, Kan., U.S.A.), 10 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.4) (Sigma) and 0.035% (w/v) sodium bicarbonate. Monolayer viability was monitored with the Trypan Blue dye exclusion test; 95% of the HeLa cells remained viable throughout experimental manipulations.

Bacteria were grown in BHI broth with shaking for 16 h at 37 °C, collected by centrifugation (6000 *g* for 10 min) and resuspended in Hanks' solution to final concentrations of 1×10^8 or 1×10^9 total bacteria ml⁻¹ for test purposes; total counts were performed with a Petroff-Hauser chamber (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.).

Reversible attachment of bacteria to HeLa cells and the role of motility during the initial phase of the interaction. A volume of bacterial suspension in Hanks' solution (1×10^9 ml⁻¹) was placed on a HeLa cell monolayer to give a layer about 1 mm deep. The preparation was incubated at 37 °C and observed by phase-contrast microscopy. Reversible attachment was defined as a form of association between bacteria and HeLa cells that was easily reversed by the fluid shear produced when five volumes of Hanks' solution was introduced on one side of the cover-slip while excess fluid was withdrawn from the opposite side. Twenty HeLa cells were examined at intervals and the average number of bacteria per HeLa cell was determined before and after fluid displacement to provide a measure of reversible attachment.

Irreversible attachment of bacteria to HeLa cells. Irreversible attachment was defined as the form of association between bacteria and HeLa cells that was not reversed by fluid displacement. Volumes (1 ml) of bacterial suspension in Hanks' solution (1×10^9 ml⁻¹) were incubated with the HeLa cell monolayers for 30 min at 37 °C in Multiplate dishes. After the removal of reversibly attached bacteria, irreversibly attached bacteria were stained with fluorescent antibody. The monolayers were first treated with rabbit antiserum prepared against boiled *S. typhimurium* cells, and then with fluorescein-conjugated goat anti-rabbit serum (Difco) for a further 15 min. Because the intact cytoplasmic membranes of animal cells are impermeable to immunoglobulins (Nairn, 1976), all the fluorescent bacteria observed on the HeLa cells were assumed to be extracellular (Kihlström & Nilsson, 1977; Hale & Bonventre, 1979). Monolayers were examined in duplicate and the numbers of bacteria associated with up to 50 HeLa cells of each monolayer were determined. Means, standard deviations (s.d.), and standard error of

means (S.E.M.) were calculated with a Wilcoxon Rank Test (Brownlee, 1965) and the aid of a computer program. Results obtained by this method were comparable to those obtained with phase-contrast microscopy.

Centrifuge-assisted attachment of *S. typhimurium* to HeLa cells. Monolayers of HeLa cells (50% confluence) were grown on circular glass cover-slips 12 mm in diameter and then placed in flat-bottomed glass vials (Rochester Scientific Co., Rochester, N.Y., U.S.A.). Each vial received 400 μ l of bacterial suspension (4×10^8 bacteria ml^{-1}) and was then centrifuged at 160 *g* for 5 min. Some cover-slips were then examined by phase-contrast microscopy for reversibly attached bacteria. The supernates of other monolayers were replaced with Hanks' solution and the monolayers were incubated for 30 min at 37 °C to allow irreversible attachment to occur. The monolayers were treated with antiserum and irreversibly attached bacteria were enumerated. These conditions were selected so that normally adhesive bacteria attached to HeLa cells in approximately equal numbers in this test and in the routine test.

Measurement of the internalization of *S. typhimurium* by HeLa cells. Volumes (1 ml) of bacterial suspension in Hanks' solution (1×10^8 ml^{-1}) were placed on each of four monolayers and incubated for 3 h. After the removal of reversibly attached bacteria, the irreversibly attached, extracellular bacteria were stained and counted as described. The total numbers of bacteria (i.e. both extracellular and intracellular populations) associated with up to 100 HeLa cells were obtained from duplicate monolayers that had been fixed in methanol for 2 min before being treated with antiserum. Fixation destroyed the selective permeability of animal cell membranes and allowed the immunoglobulins to react with intracellular bacterial antigens (Nairn, 1976; Kihlström & Nilsson, 1977; Hale & Bonventre, 1979). The invasion index represented the intracellular proportion of the total numbers of bacteria per HeLa cell, i.e.

$$\text{Invasion index} = \frac{\text{Mean count of total bacteria} - \text{Mean count of extracellular bacteria}}{\text{Mean count of total bacteria}}$$

The numbers of monolayers and HeLa cells examined, and the statistical analyses were as described above. Replication of the bacteria was estimated from comparative counts of total bacteria made at 30 min and 3 h and the invasion indices were adjusted accordingly.

Influence of fluid shear and ionic strength on attachment. Attachment was measured both in the usual fashion and after the preparations had been subjected to continual fluid shear during incubation. The latter was achieved on a rocking platform (Labindustries, Berkeley, Calif., U.S.A.) set to move through an arc of 25° at 40 cycles min^{-1} .

Attachment was measured in suspension fluids of various ionic strengths prepared from Hanks' solution diluted in isotonic sucrose buffered at pH 7.3 with 10 mM-HEPES buffer (pK_a 7.3); the final solutions were between 160 and 190 mM. The dimensions of the diffuse layer of counter ions ($1/K$) on the cell surfaces were calculated according to Heard & Seaman (1960). The theoretical distances of separation (H) between bacteria and HeLa cells at various values of $1/K$ were computed according to Weiss & Harlos (1972). The distance of closest approach between bacterium and HeLa cell was taken as that at which the potential energy of repulsion (V_R) was equal to, or less than, the potential energy of attraction (V_A) (Marshall *et al.*, 1971).

Electron microscopy. Preparations of bacteria were made on parlodian-coated grids and stained with phosphotungstic acid (2%, w/v) (Baker Chemical Co., Phillipsburg, N.J., U.S.A.) for 30 s. Specimens were examined for flagella with a Zeiss EM-10 electron microscope.

RESULTS

Reversible attachment of *S. typhimurium* to the surfaces of HeLa cells. Motile bacteria collided rapidly with the HeLa cells and such collisions often resulted in the bacteria becoming reversibly attached to the HeLa cell surface. All bacteria associated with the HeLa cells were in this state for the first 5 min of contact. Bacteria attached and detached throughout the period of observation. However, a state of equilibrium was reached within 1–2 min when 8–12 bacteria per HeLa cell were observed; this level of reversible attachment did not change appreciably for the remainder of the 30 min incubation.

Irreversible attachment of *S. typhimurium* to the surfaces of HeLa cells. After 5 min of contact, some bacteria became irreversibly attached to the HeLa cell surface and could no longer be removed by washing in Hanks' solution. Typically, 3.4 ± 0.9 (S.D.) and 4.7 ± 1.8 irreversibly attached bacteria per HeLa cell were recorded for strains TML and S850, respectively, after 5 min of incubation. The numbers of irreversibly attached bacteria increased steadily during the first 30 min when the numbers of extracellular bacteria and total bacteria associated with the HeLa cells were the same (i.e. the invasion index was zero).

Fig. 1. Time-dependent internalization of extracellular, irreversibly attached *S. typhimurium* by HeLa cells. Organisms of strain S850 (a) and strain NY (b) were grown in BHI broth, resuspended in Hanks' solution (1×10^9 ml $^{-1}$) and incubated with HeLa cell monolayers for various periods of time. The mean number of extracellular bacteria (○) and the total number of bacteria (●) per HeLa cell were determined and the standard error of the mean (vertical bar) was calculated, as described in Methods.

Table 1. *Irreversible attachment of S. typhimurium to HeLa cells and their internalization by HeLa cells*

HeLa cell monolayers were incubated with bacteria at a concentration of 1×10^9 ml $^{-1}$ for 30 min for measurement of irreversible attachment, or 1×10^8 ml $^{-1}$ for 3 h for measurement of internalization. All bacterial counts are given per HeLa cell.

Strain	Measurement of irreversible attachment	Measurement of internalization		
	Mean count ($\pm$ S.D.)	Mean extracellular count ($\pm$ S.D.)	Mean total count ($\pm$ S.D.)	Invasion index*
S850	18.4 $\pm$ 3.1	20.4 $\pm$ 6.8	55.6 $\pm$ 13.9	0.63
S2204	5.7 $\pm$ 1.8	3.3 $\pm$ 1.1	14.7 $\pm$ 5.9	0.78
TML	11.3 $\pm$ 3.3	5.1 $\pm$ 1.6	20.5 $\pm$ 5.9	0.75
W118	10.3 $\pm$ 4.9	8.6 $\pm$ 3.6	32.3 $\pm$ 6.9	0.73
NY	20.0 $\pm$ 4.4	8.5 $\pm$ 4.6	38.6 $\pm$ 8.5	0.78
PR	7.7 $\pm$ 2.0	18.9 $\pm$ 5.2	38.1 $\pm$ 8.1	0.50

* Proportion of total bacteria internalized (see Methods).

After 30 min, there was an apparent decrease in the rate at which bacteria became irreversibly attached to the HeLa cells (Fig. 1a), and in the case of strain NY alone, the numbers of attached bacteria decreased as the rate of internalization apparently exceeded the rate of attachment (Fig. 1b). In consequence, a 30 min period of incubation was selected for the measurement of the irreversible attachment. Within the limits of 1×10^8 and 1×10^9 bacteria ml $^{-1}$, the number of irreversibly attached bacteria per HeLa cell was a linear function of the concentration of bacteria (results not shown). Bacterial suspensions of concentrations of about 4×10^9 ml $^{-1}$ were unsatisfactory for two reasons: (i) the bacteria attached to the HeLa cells in aggregates that were readily dislodged, and (ii) the HeLa cell monolayers died rapidly (as judged by cell morphology and Trypan Blue dye exclusion tests). Irreversible attachment, however, was independent of the number of HeLa cells that constituted the monolayer when the latter was varied between 3×10^5 and 3×10^4 HeLa cells per monolayer. Differences in the levels of irreversible attachment of individual strains were appreciable (Table 1), but even in suspensions of the most adhesive strains, less than 1% of the total number of bacteria became attached.

Table 2. Time-dependent shift of *S. typhimurium* from an extracellular to an intracellular state

Counts of extracellular bacteria and total bacteria associated are all given per HeLa cell.

Strain	Time of incubation								
	30 min			60 min			180 min		
	Extra-cellular count	Total count	Invasion index	Extra-cellular count	Total count	Invasion index	Extra-cellular count	Total count	Invasion index
S850	14.5	14.9	0.03	18.5	47.0	0.61	34.8	98.2	0.66
S2204	4.2	3.9	0.00	5.0	23.7	0.79	22.0	43.4	0.69
TML	8.7	7.7	0.00	14.4	21.7	0.34	16.7	48.5	0.66
W118	13.3	11.3	0.00	18.6	36.4	0.49	21.0	57.7	0.64
NY	20.7	19.0	0.00	3.8	39.2	0.90	0.0	52.4	1.00
PR	6.9	7.9	0.13	13.0	30.4	0.57	20.3	59.2	0.66

Internalization of S. typhimurium by HeLa cells. All six strains had the ability to invade HeLa cells (Table 1). Observation of single monolayers that had been treated with rhodamine-conjugated antibody (Smith *et al.*, 1962) before fixation and then with fluorescein-conjugated antibody after fixation revealed distinct intracellular and extracellular populations of bacteria. The proportion of intracellular bacteria was similar to that found with the routine technique.

Internalization was time-dependent (Fig. 1*a, b*; Table 2) and reached significant proportions ($P < 0.01$ to < 0.001 ; 95% confidence limits) by 60 min for all strains except TML. Differences between the numbers of extracellular and total bacteria were highly significant ($P < 0.001$) for all strains by 3 h (Table 2) when about 60% (strains S850 and PR) or 80% (strains S2204, TML, W118 and NY) of the bacteria attached to the HeLa cells by 30 min were intracellular. A twofold or less increase in bacterial numbers occurred between 30 min and 3 h. Intracellular and extracellular bacteria appeared to replicate to about the same extent because pairs of short rods were present in similar proportions in both populations. The calculated invasion indices would be exaggerated only if the intracellular population alone had replicated. Corrections for the replication of the intracellular bacteria alone yielded invasion indices of 0.6 or greater for strains S2204, TML, W118 and NY and 0.4 for strains S850 and PR.

Influence of motility on the attachment to, and the invasion of, HeLa cells by S. typhimurium. Bacteria of motile cultures of strains TML and S850 grown in BHI broth possessed an average of six flagella and 60% or more of the bacteria were motile. Such bacteria became reversibly and irreversibly attached to the HeLa cells (Table 3) and were subsequently internalized. In contrast, bacteria grown on BHI agar produced an average of less than one flagellum and less than 1% of the bacteria were motile. Bacteria grown on BHI agar failed to become either reversibly or irreversibly attached to HeLa cells (Table 3) and no intracellular bacteria were observed. Broth-grown cultures of non-motile, non-flagellate mutants of strains TML and S850 behaved in an identical manner to agar-grown cultures of the parental strains (Table 3). The reversion of the non-motile mutants to a motile state coincided with the reappearance of reversible and irreversible attachment (Table 3) and invasive properties.

However, non-motile bacteria of both strains did become attached to HeLa cells when impacted on to the HeLa cells by centrifugation. Initially, large numbers of bacteria (i.e. > 50 bacteria per HeLa cell) were reversibly attached to the HeLa cell surfaces but subsequent incubation of the monolayers resulted in only some of these bacteria becoming irreversibly attached (Table 3). Bacteria that became irreversibly attached to the HeLa cells after centrifugation were also internalized by the HeLa cells.

Table 3. *Promotion of reversible and irreversible attachment of S. typhimurium to HeLa cells by motility and centrifugation*

Counts of reversibly and irreversibly attached bacteria are all given per HeLa cell.

Strain	Origin	Remarks	Motility (%)	Influence of motility		Centrifuge-assisted attachment
				Reversibly attached bacteria*	Irreversibly attached bacteria ($\pm$ s.d.)	Irreversibly attached bacteria ($\pm$ s.d.)†
S850	Wild-type	Grown in BHI broth	70	9.7	21.6 $\pm$ 5.9	20.6 $\pm$ 4.3
S850	Wild-type	Grown on BHI agar	<1	0.1	2.1 $\pm$ 0.9	18.0 $\pm$ 5.1
TML	Wild-type	Grown in BHI broth	60	10.1	12.6 $\pm$ 2.9	11.8 $\pm$ 2.2
TML	Wild-type	Grown on BHI agar	<1	0.2	0.9 $\pm$ 0.8	10.3 $\pm$ 3.7
S850	Parent	Motile wild-type	70	9.1	18.4 $\pm$ 5.8	19.3 $\pm$ 4.1
S850(NM4)	S850	Non-motile mutant	0	0.0	1.2 $\pm$ 1.1	16.0 $\pm$ 3.1
TML	Parent	Motile wild-type	60	10.0	10.4 $\pm$ 2.2	12.1 $\pm$ 2.2
TML(NM3)	TML	Non-motile mutant	0	0.0	1.2 $\pm$ 1.2	10.2 $\pm$ 2.2
S850	Parent	Motile wild-type	70	11.2	19.1 $\pm$ 4.7	21.6 $\pm$ 5.1
S850(NM4R6)	S850(NM4)	Motile revertant	80	10.4	17.7 $\pm$ 3.6	22.4 $\pm$ 3.9
TML	Parent	Motile wild-type	60	10.2	10.6 $\pm$ 1.9	13.1 $\pm$ 3.1
TML(NM3R1)	TML(NM3)	Motile revertant	80	11.3	11.5 $\pm$ 1.7	13.1 $\pm$ 4.6

* Mean of five consecutive observations.

† Mean number of reversibly attached bacteria >50.

Fig. 2. Influence of the dimension of the diffuse double layers on the reversible (—) and irreversible (---) attachment of bacteria to HeLa cells. Organisms of strains S850 (●) and TML (○) were grown in BHI broth and suspended in a sucrose/HEPES buffer solution to which various quantities of Hanks' solution were added to give a total of 160 mmol l⁻¹, but different ionic strengths. The dimensions of the diffuse double layers (1/K) were calculated from the ionic strengths (I) with expression of Heard & Seaman (1960): $1/K = 3.05I^{-1/2}$.

Influence of reversible attachment on the irreversible attachment of bacteria to HeLa cells. Fluid shear produced by rocking the incubation mixtures prevented all reversible attachment and reduced irreversible attachment by approximately 90%. Fluid displacement, however, failed to remove bacteria that had become irreversibly attached prior to the application of the shear force.

Influence of ionic strength on attachment. The calculated value of 1/K for Hanks' solution was 0.72 nm. Increases in the value of 1/K resulting from decreasing the ionic strength caused a concomitant decrease in the numbers of bacteria irreversibly attached to the HeLa cells; minimal values of 1–2 bacteria per HeLa cell were reached when 1/K was about 2.5 nm (Fig. 2). Only rare HeLa cells (<1% of the total) with an array of fine filaments over their

entire surfaces allowed significant irreversible attachment (10–20 bacteria per cell) to occur when diluted Hanks' solution was used ($1/K$ 4.12 nm). Once irreversibly attached, the bacteria were not dislodged from the HeLa cell surface by solutions of low ionic strength. The reversible attachment of bacteria was unaltered by $1/K$ values up to 4.12 nm (Fig. 2).

The theoretical distances of separation (H) between the bacteria and the HeLa cells for these values of $1/K$ were calculated with the following dimensions: HeLa cell surface potential, -14 mV [calculated (Ambrose, 1966) with the data of Sachtleben & Luyken (1962) and Fuhrman (1965)]; bacterial cell surface potential, -25 mV [calculated (Ambrose, 1966) from the values given by James (1957)]; HeLa cell radius of curvature, 1×10^{-3} cm (Weiss & Harlos, 1972); bacterial cell radius of curvature, 4×10^{-5} cm (Marshall *et al.*, 1971); dielectric constant, 81.07 (of water); Hamaker constant, 1×10^{-4} ergs [estimated from the data of Wilkins *et al.* (1962)]. When $1/K$ was 2.5 nm, the distance of separation was about 14 nm. At $1/K$ values of 0.72 and 4.12 nm, these distances were about 3 and 26 nm, respectively.

Deviations from these values of H caused by variations in the other parameters were computed over the following ranges: HeLa cell surface potential, -10 to -20 mV (Jones, 1977); HeLa cell radius of curvature, 1×10^4 cm (planar surface) to 5×10^{-6} cm [the estimated size of filaments on the surfaces of HeLa cells (Weiss & Harlos, 1972)]; dielectric constant, 70 to 80 [that expected for a mixture of sucrose and dilute salt solutions (Pollack *et al.*, 1965)]. When $1/K$ was 2.5 nm, H was 13.0 to 15.0 nm. Only the use of the hypothetical Hamaker constant of 1×10^{-16} ergs (Weiss & Harlos, 1972) caused H to increase substantially to 28.0 nm when $1/K$ equalled 2.5 nm.

Neither the molarity of the suspending fluid (160–300 mmol l^{-1}) nor the presence of sucrose influenced irreversible attachment at ionic strengths equal to that of Hanks' solution. Higher molarities impaired motility and attachment was curtailed. Reduced irreversible attachment resulted in a parallel decrease in the numbers of bacteria internalized.

DISCUSSION

The relevance of the interactions of *S. typhimurium* with HeLa cells to the behaviour of the bacteria in natural surroundings is a matter of conjecture at present. The model system, however, allowed possibly significant interactions to be identified. The most pertinent of these were probably irreversible attachment and internalization. Motility appeared only to promote contact between the bacterium and animal cell which initiated the sequence. Reversible attachment, however, was a prerequisite of irreversible attachment to the extent that continual fluid displacement prevented reversible attachment and this prevented, but did not reverse, irreversible attachment. It was inferred, therefore, that the period of reversible attachment provided the time necessary for a putative bacterial adhesin to bind to the HeLa cell surface.

The two forms of attachment appeared to be caused by different types of attractive forces generated between the bacterial and animal cell surfaces. The derivation of the so-called DLVO theory employed (Weiss & Harlos, 1972) is applicable only to the analysis of weak long-range interactions of the reversible type (Marshall *et al.*, 1971). Briefly, the theory assumes that the forces of attraction (V_A) and the forces of repulsion (V_R) generated between two surfaces are additive, with states of mutual attraction occurring at two distances of separation when $V_A > V_R$. At long range, the weak attractive forces are those of the secondary minimum whereas at shorter distances of separation the stronger forces of attraction at the primary minimum become apparent. At a distance intermediate between these, the interaction of the ionic clouds of the diffuse double layers causes mutual repulsion between the bodies when $V_R > V_A$. It has been shown that the latter is sufficient to prevent physical contact between a bacterium and a surface (Marshall *et al.*, 1971). The stronger binding forces equivalent to those of the primary minimum, therefore, may occur only through the agencies of long filamentous appendages (or adhesins) on the bacterial cell

binding to the substratum (Jones, 1977). A decrease in the ionic strength of the suspending fluid increases the dimensions of the diffuse double layers ($1/K$) and causes increased separation of the surfaces, perhaps to the extent that all interactions at the secondary minimum are eliminated (Marshall, 1975) and all attachment is prevented (Marshall *et al.*, 1971). In the present studies only irreversible attachment was inhibited when H measured 15.0 to 26.0 nm. These results may be interpreted as follows: reversible attachment was caused by long-range forces of the secondary minimum; irreversible attachment was probably due to the stronger binding forces of the primary minimum and hence must have occurred via the agency of a salmonella adhesin. The calculations were based initially on the assumption that the HeLa cell surfaces were near planar whereas HeLa cells do produce filamentous surface projections (Fisher & Cooper, 1967; Pugh-Humphreys & Sinclair, 1970; Kihlström & Latkovic, 1978). Accommodation of this variable and others, however, did not appreciably alter the value of H or the conclusions.

The conclusion that the close juxtaposition of bacterium with animal cell occurs prior to invasion is clearly supported by the observations of Kihlström & Nilsson (1977) on the invasion of HeLa cells by rough mutant salmonellae, but not by the observations of Takeuchi (1967) on the invasion of epithelial cells by strain W118. However, neither the extensive glycocalyx surface of the epithelial cells (Ito, 1969), nor the extensive lipopolysaccharide layer of the bacteria (Shands, 1965) are apparent from the photomicrographs of Takeuchi (1967) and an estimation of the true distance of separation is impossible.

For two reasons the adhesins involved are unlikely to be type 1 fimbriae. Firstly, *S. typhimurium* strains S850 and S2204 belonged to the non-fimbriate FIRN biogroup (Duguid *et al.*, 1975). Secondly, the gap of 15.0 nm between bacterium and HeLa cell (which prevented irreversible attachment) represents less than 1% of the typical length of type 1 fimbriae. If the binding sites of type 1 fimbriae occurred along the lateral margin (Salit & Gotschlich, 1977; Sweeney & Freer, 1979) rather than the terminus (Brinton, 1965) then fully 99% of the fimbrial length would need to be employed in binding to the HeLa cell surface to account for these results. Thus, two alternatives may be assumed: (i) salmonellae of the FIRN biogroup with a common genetic ancestry (Old & Duguid, 1979) have evolved a different but common adhesive mechanism, whereas strains not belonging to the FIRN biogroup utilize type 1 fimbriae; (ii) all adhesive salmonellae produce a similar adhesive material which is distinct from type 1 fimbriae. The latter need not involve the hydrophobic surface properties associated with the adhesion of rough mutants (Kihlström, 1980), although such bacteria would associate with the surface of an animal cell more readily (Van Oss, 1978). All six strains examined in the present study had a smooth cell wall composition according to phage sensitivity tests.

Microbial replication during the 3 h of incubation was similar to that found by others (Lowrie *et al.*, 1979) and tended to obfuscate the results. However, significant proportions of irreversibly attached bacteria entered the animal cells between 30 min and 3 h. In one case (strain NY), the rate of internalization appeared to greatly exceed the rate of attachment with the result that 90–100% of the bacteria became intracellular. Only irreversibly attached bacteria entered the HeLa cells. This mode of attachment appeared to be a critical part of the internalization process and its nature, accordingly, would appear to be of consequence to the bacterium in its role as an intracellular parasite.

We are indebted to Dr R. Freter of this department for valuable discussions and for help with the computations. This work was supported by Public Health Service Grant AI-13842 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

AMBROSE, E. J. (1966). Electrophoretic behavior of cells. In <i>Progress in Biophysics and Molecular</i>	<i>Biology</i>, vol. 16, pp. 243–265. Edited by J. A. V. Butler & H. E. Huxley. Oxford: Pergamon Press.

- BRINTON, C. C. (1965). Structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram negative bacteria. *Transactions of the New York Academy of Sciences, Series II* **27**, 1003–1054.
- BROWNLIE, K. A. (1965). *Statistical Theory and Methodology in Science and Engineering*. London & New York: John Wiley.
- CAPO, C., BONGRAND, P., BENOLIEL, A. M. & DEPIEDS, R. (1978). Dependence of phagocytosis on strengths of phagocyte-particle interaction. *Immunology* **35**, 117–182.
- DUGUID, J. P., ANDERSON, E. S., ALFREDSSON, G. A., BARKER, R. & OLD, D. C. (1975). A new biotyping scheme for *Salmonella typhimurium* and its phylogenetic significance. *Journal of Medical Microbiology* **8**, 149–166.
- FISHER, H. W. & COOPER, T. W. (1967). Electron-microscope studies of the microvilli of HeLa cells. *Journal of Cell Biology* **34**, 569–576.
- FRETER, R., O'BRIEN, P. C. M. & MACSAL, M. S. (1979). Effect of chemotaxis on the interaction of cholera vibrios with intestinal mucosa. *American Journal of Clinical Nutrition* **32**, 128–132.
- FUHRMAN, G. F. (1965). Cytopherograms of normal, proliferating and malignant liver cells. In *Cell Electrophoresis*, pp. 92–98. Edited by E. J. Ambrose. Boston: Little, Brown and Co.
- GIANNELLA, R. A., WASHINGTON, O., GEMSKI, P. & FORMAL, S. B. (1973). Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of salmonella. *Journal of Infectious Diseases* **128**, 69–75.
- GRIFFIN, F. M., GRIFFIN, J. A., LEIDER, J. E. & SILVERSTEIN, S. C. (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *Journal of Experimental Medicine* **142**, 1263–1282.
- HALE, T. L. & BONVENTRE, P. F. (1979). Shigella infection of Henle intestinal epithelial cells: role of the bacterium. *Infection and Immunity* **24**, 879–886.
- HEARD, D. H. & SEAMAN, G. V. F. (1960). The influence of pH and ionic strength on electrokinetic stability of the human erythrocyte membrane. *Journal of General Physiology* **43**, 635–654.
- ITO, S. (1969). Structure and function of the glycocalyx. *Federation Proceedings* **28**, 12–25.
- JAMES, A. M. (1957). The electrochemistry of the bacterial surface. In *Progress in Biophysics and Biophysical Chemistry*, vol. 8, pp. 96–142. Edited by J. A. V. Butler & B. Katz. New York & London: Pergamon Press.
- JONES, G. W. (1977). The attachment of bacteria to the surfaces of animal cells. In *Microbial Interactions. Receptors and Recognition, Series B*, vol. 3, pp. 139–176. Edited by J. L. Reissig. London & New York: Chapman & Hall.
- JONES, G. W. & FRETER, R. (1976). Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush-border membranes and human erythrocytes. *Infection and Immunity* **14**, 240–245.
- KIHLSTRÖM, E. (1980). Interaction between salmonella bacteria and mammalian non-professional phagocytes. *American Journal of Clinical Nutrition* **33**, 2491–2501.
- KIHLSTRÖM, E. & EDEBO, L. (1976). Association of viable and inactivated *Salmonella typhimurium* 395MS and MR10 with HeLa cells. *Infection and Immunity* **14**, 851–857.
- KIHLSTRÖM, E. & LATKOVIC, S. (1978). Ultrastructural studies on the interaction between *Salmonella typhimurium* 395MS and HeLa cells. *Infection and Immunity* **22**, 804–809.
- KIHLSTRÖM, E. & NILSSON, L. (1977). Endocytosis of *Salmonella typhimurium* 395MS and MR10 by HeLa cells. *Acta pathologica et microbiologica scandinavica* **B85**, 322–328.
- LOWRIE, D. B., ABER, V. R. & CARROL, M. E. W. (1979). Division and death rates of *Salmonella typhimurium* inside macrophages: use of penicillin as a probe. *Journal of General Microbiology* **110**, 409–419.
- MARSHALL, K. C. (1975). Clay mineralogy in relation to survival of soil bacteria. *Annual Review of Phytopathology* **13**, 357–373.
- MARSHALL, K. C., STOUT, R. & MITCHELL, R. (1971). Mechanism of the initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology* **68**, 337–348.
- NAIRN, R. C. (editor) (1976). *Fluorescent Protein Tracing*, 4th edn. Edinburgh & London: Livingston.
- OLD, D. C. & DUGUID, J. P. (1979). Transduction of fimbriation demonstrating common ancestry in FIRN strains of *Salmonella typhimurium*. *Journal of General Microbiology* **112**, 251–259.
- POLLACK, W., HAGER, H. J., RECKEL, R., TOREN, D. A. & SINGHER, H. O. (1965). A study of the forces involved in the second stage of haemagglutination. *Transfusion* **5**, 158–183.
- PUGH-HUMPHREYS, R. G. P. & SINCLAIR, W. (1970). Ultra-structural studies relating to the surface morphology of cultured cells. *Journal of Cell Science* **6**, 477–484.
- SACHTLEBEN, P. & LUYKEN, R. (1962). Veränderungen des elektrokinetischen Potentials von HeLa-Zellen nach Infektion mit Poliomyelitis Virus. *Archiv für die gesamte Virusforschung* **11**, 732–742.
- SALIT, I. E. & GOTSCHLICH, E. (1977). Type 1 *Escherichia coli* pili: characterization of binding to monkey kidney cells. *Journal of Experimental Medicine* **146**, 1182–1194.
- SHANDS, J. W. (1965). Localization of somatic antigen on gram-negative bacteria by electron microscopy. *Journal of Bacteriology* **90**, 266–270.
- SILVERSTEIN, S. C., STEINMAN, R. M. & COHN, Z. A. (1977). Endocytosis. *Annual Review of Biochemistry* **46**, 669–722.
- SMITH, M. L., CARSKI, T. R. & GRIFFIN, C. W. (1962). Modification of fluorescent-antibody procedures employing crystalline tetramethylrhodamine isothiocyanate. *Journal of Bacteriology* **83**, 1358–1359.
- STOSSEL, T. P. (1975). Phagocytosis: recognition and ingestion. *Seminars in Hematology* **12**, 83–116.
- SWEENEY, G. & FREER, J. H. (1979). Location of binding sites on common type 1 fimbriae from *Escherichia coli*. *Journal of General Microbiology* **112**, 321–328.
- TAKEUCHI, A. (1967). Electron microscope studies of

- experimental salmonella infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *American Journal of Pathology* **50**, 109–136.
- VAN OSS, C. J. (1978). Phagocytosis as a surface phenomenon. *Annual Review of Microbiology* **32**, 19–39.
- WEISS, L. & HARLOS, J. P. (1972). Short-term interactions between cell surfaces. In *Progress in Surface Science*, vol. 1, pp. 355–405. Edited by S. G. Davison. Oxford: Pergamon Press.
- WILKINS, D. J., OTTEWILL, R. H. & BANGHAM, A. D. (1962). On the flocculation of sheep leucocytes: II. Stability studies. *Journal of Theoretical Biology* **2**, 176–191.
- WILKINSON, R. G., GEMSKI, P. & STOCKER, B. A. D. (1972). Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. *Journal of General Microbiology* **70**, 527–554.