The Attachment to, and Invasion of HeLa Cells by *Salmonella typhimurium*: The Contribution of Mannose-sensitive and Mannose-resistant Haemagglutinating Activities

By G. W. JONES* AND LEIGH A. RICHARDSON

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

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The association of the haemagglutinating activities of *Salmonella typhimurium* cultures with bacterial adhesion to HeLa cells, and the internalization of the bacteria by HeLa cells, was studied. Adhesion was not inhibited by α -methyl-D-mannoside (i.e. adhesion was mannose-resistant), and only four of the six strains tested produced type 1 fimbriae and the associated mannose-sensitive haemagglutinin (MSHA). The other two strains belonged to the non-fimbriate FIRN biogroup. Cultures of all six strains contained a mannose-resistant haemagglutinating (MRHA) activity when grown at 37 °C, but cultures of only one fimbriate and one non-fimbriate strain did so when grown at 18 °C. From the comparison of cultures grown at 18 °C and 37 °C, and of mutant strains with the phenotypes MRHA-negative/MSHA-positive, or MRHA-positive/MSHA-negative, it was concluded that the MRHA activity was responsible for the attachment of salmonellae to HeLa cells. Only bacterial adhesion that was resistant to mannose resulted in the internalization of the bacteria by the HeLa cells.

INTRODUCTION

Of the many investigations of invasive intracellular pathogens and their host cells [for example, salmonellae – Takeuchi (1967), Giannella *et al.* (1973), Kihlström & Edebo (1976), Kihlström & Latkovic (1978) and Carrol *et al.* (1979); shigellae – LeBrec *et al.* (1964), Takeuchi *et al.* (1965), Gemski & Formal (1975), Hale & Bonventre (1979) and Hale *et al.* (1979); mycobacteria – Hart *et al.* (1972); chlamydiae – Becker (1978), Byrne & Moulder (1978), Brownridge & Wyrick (1979) and Gregory *et al.* (1979); toxoplasma – Norrby & Lycke (1967) and Jones & Hirsch (1972)], few have been concerned with the role of the bacterium prior to and during the initial phase of internalization. Pertinent studies of these early events by Kihlström & Edebo (1976) established that *Salmonella typhimurium* attached to HeLa cells prior to their internalization and that attachment was greatest when a rough mutant *S. typhimurium* was employed. More recently, it has been shown that smooth *S. typhimurium* also readily attach to HeLa cells. The process involved an initial phase of reversible attachment which was followed by the essential phase of irreversible attachment (Jones *et al.*, 1981) prior to the internalization of the bacteria by the HeLa cells. Irreversible attachment possibly mediated by bacterial adhesins (Jones *et al.*, 1981) may then have triggered the internalization of the bacteria in ways similar to those necessary for the internalization of particles by phagocytes (Stossel, 1975; Silverstein *et al.*, 1977).

The most apparent adhesive activities of cultures of *S. typhimurium* are those due to the presence of type 1 fimbriae (Duguid & Old, 1980), which facilitate the attachment of the bacteria to a variety of surfaces and cause haemagglutination. All forms of attachment mediated by these organelles are inhibited in the presence of D-mannose (Duguid & Old, 1980).

However, *S. typhimurium* strains of the non-fimbriate FIRN biogroup (Duguid *et al.*, 1975) also adhere to HeLa cells (Jones *et al.*, 1981). The possibility existed, therefore, that the attachment of *S. typhimurium* to HeLa cells was the result of an adhesive activity perhaps common to all *S. typhimurium* and hence not that mediated by type 1 fimbriae. Haemagglutinating activities may reflect adhesive mechanisms essential for the survival of bacteria in natural habitats (Jones, 1977; Beachey, 1980). Accordingly, both the mannose-sensitive haemagglutinin (MSHA) of type 1 fimbriae and possible mannose-resistant haemagglutinins (MRHA) of *S. typhimurium* cultures were investigated, and their role in enabling the bacteria to attach to, and invade HeLa cells was studied.

METHODS

Bacterial strains and cultures. Six wild-type strains described previously (Jones *et al.*, 1981) and one laboratory strain derived from LT2 were used (Table 1). Bacteria were grown routinely in Brain-Heart Infusion (BHI) broth (Difco) at 37 °C (Jones *et al.*, 1981). For some experiments, cultures were grown on BHI agar (Difco) at 37 °C or in BHI broth at 18 °C. Incubation was for 16–24 h.

Test for mannose-sensitive haemagglutination (MSHA). MSHA activity was used as an indicator of type 1 fimbriae production. The test used was a modified form of the tile test described by Duguid *et al.* (1966). In this test, haemagglutination is a function of both the bacterial numbers and the time for which the bacteria and erythrocytes are in contact and therefore standard curves can be constructed from plots of these two parameters. Suspensions containing various concentrations of the reference fimbriate strain TML together with the non-fimbriate strain S850 were prepared in saline (0.85%, w/v, NaCl) such that the final bacterial concentration was always 5×10^{10} ml⁻¹. Portions (25 µl) of these bacterial suspensions were then mixed on a mechanical shaker with 2 vol. of a 1.5% (v/v) suspension of methyl glyoxal-treated horse erythrocytes (Hirata & Brandriss, 1968) in saline, and the time required for perceptible haemagglutination to occur was recorded. A standard curve was then constructed. Test bacteria were suspended in saline at a concentration of 5×10^{10} ml⁻¹ and their haemagglutinating activity was measured as a function of time. Relative MSHA activities were then obtained by extrapolation from the standard curve. Cultures with a relative MSHA activity of 1.0 (equivalent to an undiluted suspension of strain TML at a concentration of 5×10^{10} bacteria ml⁻¹) agglutinated the erythrocytes within 5 s whereas cultures with the lowest measurable activity (a relative MSHA activity of 0.01 and equivalent to a 1 in 100 dilution of suspension of strain TML) caused haemagglutination in 3 min. The mannose sensitivities of the haemagglutinins were measured in tests to which 0.1% (w/v) D-mannose (Pfanstiehl Laboratories, Waukegan, Ill., U.S.A.) was added.

Test for mannose-resistant haemagglutination (MRHA). MRHA activity was detected with a micro-haemagglutination technique which did not detect mannose-sensitive haemagglutinins (Jones & Rutter, 1974). Bacteria were suspended in saline at a concentration of 5×10^{10} ml⁻¹. A series of twofold dilutions were prepared in saline and in saline containing 0.2% (w/v) D-mannose, and equal volumes of a 1% (v/v) suspension of methyl glyoxal-treated sheep erythrocytes in saline were added. Tests were incubated in an ice-bath for 2 h. The haemagglutination titre was recorded as the reciprocal of the highest dilution of bacterial culture to cause complete haemagglutination in the presence of D-mannose (Jones & Rutter, 1974).

Interactions of *S. typhimurium* and HeLa cells. The ability of *S. typhimurium* to irreversibly attach to HeLa cells and to be internalized by HeLa cells was measured as described by Jones *et al.* (1981). Irreversible attachment or adhesion was expressed as an adhesion index, i.e. the mean number of irreversibly attached salmonellae per HeLa cell. Bacteria in Hanks' Balanced Salt solution (1×10^9 ml⁻¹) were incubated for 30 min at 37 °C with HeLa cell monolayers. The monolayers were then washed and treated with fluorescent antibody. The bacteria attached to 20–50 HeLa cells on each of two monolayers were counted. The mannose sensitivity of adhesion was measured in tests to which α-methyl-D-mannose or D-mannose (Pfanstiehl Laboratories) were added to final concentrations up to 1% (w/v). The invasion index represented the proportion of the irreversibly attached salmonellae that had entered the HeLa cells after 3 h incubation. All analyses and interpretations were as described by Jones *et al.* (1981).

Isolation of mutants constitutive for type 1 fimbriae and MSHA production. Bacteria treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG, Sigma) as described (Jones & Freter, 1976) were grown on BHI agar plates and then passed five times on the same medium to repress fimbriae production. The bacteria were then harvested in saline, mixed with a 3% (v/v) suspension of methyl glyoxal-treated horse erythrocytes and the mixtures were agitated to allow fimbriate bacteria to attach to the erythrocytes. The washed erythrocytes were then cultured on BHI agar and individual colonies were examined for MSHA activity. MSHA-positive colonies were subcultured 12 times on dry BHI agar plates and retested. Isolates [TML(AU6) and TML(AN2); Table 1] with relative MSHA activities of 1.0 to 0.9 after growth on BHI agar for this number of passages were characterized as constitutive mutants not subject to the typical phase variation of fimbriae production (Ottow,

Table 1. *Relevant properties of the strains of Salmonella typhimurium studied*

Strain	Relevant properties	Source/Reference(s)
<i>Wild-type</i>		
S850 } S2204 }	FIRN biogroup, MSHA-negative, MRHA-positive, attach to HeLa cells, invasive	Duguid <i>et al.</i> (1975), Jones <i>et al.</i> (1981)
TML } W118 }	Non-FIRN biotypes, MSHA-positive, MRHA-positive, attach to HeLa cells, invasive	Giannella <i>et al.</i> (1973), Jones <i>et al.</i> (1981), Takeuchi (1967)
NY } PR } 18 }	Non-FIRN biotypes, MSHA-positive, MRHA-positive, attach to HeLa cells, invasive MSHA-positive, derivative of strain LT2	Obtained from E. J. Gangarosa, Jones <i>et al.</i> (1981) Obtained from E. Jackson
<i>Mutants</i>		
TML(AU6) } TML(AN2) }	Constitutive MSHA-positive forms of strain TML, otherwise as strain TML	See text
TML(Y42) } TML(N12) }	MSHA-negative mutants of strain TML, otherwise as TML	See text
TML(N15)	MSHA-positive revertant of strain TML(N12), as strain TML	See text
TML(16) } TML(54) } TML(72) }	MRHA-negative mutants of strain TML, no attachment to HeLa cells, not invasive, otherwise as strain TML	See text
TML(161A)	MRHA-positive revertant of strain TML(16), as strain TML	See text
<i>Transductants</i>		
S850(PR22T) } S850(PR16T) }	MSHA-positive transductants of strain S850 produced with P22 phage lysates of strain PR, otherwise as strain S850	See text

1975). Wild-type strains exhibited typical phase variation and failed to produce the MSHA after growth on agar for one to two passages.

Isolation of MSHA-negative mutants. Cultures mutagenized with NTG were grown in BHI broth to bring about maximal expression of the MSHA (see Results). Bacteria were collected by centrifugation, suspended in saline and then mixed with a 3% (v/v) suspension of methyl glyoxal-treated horse erythrocytes to remove haemagglutinating bacteria. The bacteria not removed by the erythrocytes were recovered and grown in BHI broth. The process was repeated until the resulting cultures were only weakly haemagglutinating. Individual colonies were grown in broth and examined for MSHA activity. MSHA-negative isolates were designated TML(Y42) and TML(N12) (Table 1). An MSHA-positive revertant, designated TML(N15) (Table 1), was selected from passaged static broth cultures of strain TML(N12).

Isolation of MRHA-negative mutants. Bacteria were treated with u.v. light and grown in BHI broth to produce cultures of maximum MRHA content (see Results). The resulting cultures were repeatedly fractionated on phenyl-Sepharose (Pharmacia) columns (Smyth *et al.*, 1978). On each occasion non-motile organisms were removed from the unadsorbed fraction by selecting for motility on semi-solid agar (Freter *et al.*, 1979) before being grown in BHI broth and again subjected to chromatography. Single-colony isolates were examined for MRHA activity. MRHA-negative isolates were designated TML(16), TML(54) and TML(72) (Table 1), and a spontaneous MRHA-positive revertant isolated from a stored culture of strain TML(16) was designated TML(161A) (Table 1).

Isolation of MSHA-positive transductants of FIRN biotype strain S850. The technique was essentially similar to that described by Old & Duguid (1971). Phage lysates prepared with bacteriophage P22C_{HT} (supplied by Dr Ethel Jackson of this department) and strains 18 or PR were used to infect cultures of strain S850. Infected cultures were grown in BHI broth for the selection of fimbriate forms (Old & Duguid, 1971) and cultures that exhibited haemagglutinating activity with horse erythrocytes were plated on MacConkey agar base containing 1% (w/v) L-rhamnose (Sigma). Individual rhamnose-negative colonies of strain S850 were subcultured in broth and the relative MSHA activities were measured. MSHA-positive transductants used in the present study were designated S850(PR16T) and S850(PR22T) (Table 1).

Additional characterization of S. typhimurium strains. Cultures were tested for O and H antigens with specific antisera (Difco), for the loss of cell-wall components with rough- and smooth-specific phage (Wilkinson *et al.*, 1972), for motility and for their ability to reversibly attach to HeLa cells (Jones *et al.*, 1981). All cultures used in this study were motile (unless grown on agar), resistant to rough-specific phage, and if motile, able to reversibly attach to HeLa cells. The first and last properties were known to be important prerequisites for adhesion and internalization of the bacteria by the HeLa cells (Jones *et al.*, 1981). Electron microscopic studies were conducted on preparations of bacteria made on parlodian-coated grids (Jones *et al.*, 1981).

Table 2. *Haemagglutinating and adhesive properties of wild-type S. typhimurium strains*

Strain	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ S.D.)	
			α -MM* absent	α -MM* present
S850	0.00	16	18.4 $\pm$ 3.1	18.2 $\pm$ 4.1
S2204	0.00	4	5.8 $\pm$ 2.0	5.6 $\pm$ 1.8
TML	1.00	16	10.4 $\pm$ 2.3	12.6 $\pm$ 2.3
W118	0.95	16	15.2 $\pm$ 4.1	16.1 $\pm$ 3.8
NY	1.00	16	19.6 $\pm$ 2.8	21.4 $\pm$ 2.9
PR	1.00	8	11.1 $\pm$ 3.1	11.9 $\pm$ 2.7

* α -MM, α -Methyl-D-mannoside.

RESULTS

Haemagglutinating and adhesive properties of S. typhimurium strains

Wild-type strains. MSHA activity was detected with the tile test in cultures of four of the six wild-type strains of *S. typhimurium* tested (Table 2). The haemagglutinins were most active on guinea-pig, horse and chicken erythrocytes and active to a lesser extent on sheep, cow and human erythrocytes. Treatment of the erythrocytes with methyl glyoxal had no noticeable effect on the haemagglutination reaction. The haemagglutinin content of cultures grown at 37 °C reached near maximum levels after 16–24 h growth. Little or no MSHA activity was detected in cultures grown on BHI agar and no MRHA activity was detected. On the basis of these observations the MSHA activity was ascribed to the production of type 1 fimbriae (reviewed by Duguid & Old, 1980). The existence in haemagglutinating cultures of fimbriae similar in size to type 1 fimbriae (Duguid *et al.*, 1966) was confirmed by electron microscopy. Neither MSHA activity nor structures resembling type 1 fimbriae were detected in cultures of strains S850 and S2204.

MRHA activity was detected in broth- and agar-grown cultures of all six strains with the microhaemagglutination test (Table 2). The MRHA activity was similar with untreated and methyl glyoxal-treated erythrocytes of chicken, goose, guinea-pig, horse, cow, human and sheep and was greatest when tests were incubated in an ice-bath. Maximum MRHA titres of 8–32 were obtained with methyl glyoxal-treated sheep erythrocytes and 16–24 h old BHI broth cultures. The lowest dilution of bacterial suspension examined was a dilution of 1 in 2 with a bacterium to erythrocyte ratio of about 500 to 1. The absence of MRHA in a culture was therefore recorded as <2 .

Wild-type MSHA-positive strains of *S. typhimurium* were no more adhesive than the wild-type MSHA-negative strain S850, and in no instance was the adhesion index of an MSHA-positive strain reduced by the presence of D-mannose or α -methyl-D-mannoside (1%, w/v, or less) (Table 2). On the contrary, the presence of these carbohydrates occasionally elevated adhesion above that of bacteria tested in Hanks' solution. D-Mannose and α -methyl-D-mannoside had no effect on the adhesion of non-fimbriate bacteria to HeLa cells (Table 2).

Mutants constitutive for MSHA production. Broth-grown cultures of the parental strain TML and the two constitutive mutant strains TML(AU6) and TML(AN2) were motile and showed both MSHA and MRHA activity. When grown on BHI agar, all three strains had MRHA activity and were non-motile (Table 3), but differed in that strain TML failed to produce the MSHA under these conditions. The adhesion indices of all broth-grown cultures were similar and in no instance were these significantly reduced by the addition of 0.1% (w/v) α -methyl-D-mannoside to the reaction mixtures (Table 3). TML organisms grown on BHI agar did not attach to HeLa cells (Table 3) because the bacteria were non-motile (Jones *et al.*, 1981). Agar-grown organisms of strains TML(AU6) and TML(AN2), however, did

Table 3. *Haemagglutinating and adhesive properties of MSHA-positive constitutive mutants*

Strain	Remarks	Motility (%)	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ S.D.)		Invasion index*
					α -MM absent	α -MM present	
<i>Growth in broth</i>							
TML	Wild-type	60	1.0	16	14.1 $\pm$ 2.9	15.3 $\pm$ 3.1	0.69 ^a
TML(AU6)	Constitutive MSHA-positive mutant	70	1.0	8	15.3 $\pm$ 3.0	14.6 $\pm$ 3.2	0.73 ^a
TML(AN2)	Constitutive MSHA-positive mutant	70	1.0	4	12.7 $\pm$ 1.6	12.7 $\pm$ 2.0	0.78 ^a
<i>Growth on agar</i>							
TML	Wild-type	<1	$\leq$ 0.01	8	1.8 $\pm$ 1.7	1.1 $\pm$ 1.3	0.05 ^b
TML(AU6)	Constitutive MSHA-positive mutant	<1	1.0	4	5.6 $\pm$ 0.8	0.6 $\pm$ 1.3	0.10 ^b
TML(AN2)	Constitutive MSHA-positive mutant	<1	0.9	4	4.8 $\pm$ 1.3	1.1 $\pm$ 0.9	0.14 ^b

* Proportion of attached bacteria internalized (Jones *et al.*, 1981): *a*, significant proportion internalized ($P < 0.001$); *b*, proportion internalized insignificant ($P > 0.05$).

Table 4. *Haemagglutinating and adhesive properties of MSHA-negative mutants*

Strain	Remarks	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ S.D.)	
				α -MM absent	α -MM present
TML	MSHA-positive wild-type	1.0	8	10.4 $\pm$ 3.2	11.9 $\pm$ 3.0
TML(Y42)	MSHA-negative mutant	0.0	4	10.4 $\pm$ 2.6	10.4 $\pm$ 2.1
TML(N12)	MSHA-negative mutant	0.0	4	9.9 $\pm$ 2.6	9.2 $\pm$ 2.0
TML(N15)	MSHA-positive revertant	0.9	4	6.6 $\pm$ 2.9	8.5 $\pm$ 2.4

Table 5. *Haemagglutinating and adhesive properties of MSHA-positive transductants*

Strain	Remarks	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ S.D.)	
				α -MM absent	α -MM present
S850	Recipient	0.0	8	15.8 $\pm$ 3.4	15.9 $\pm$ 3.1
PR	Donor	1.0	4	10.8 $\pm$ 3.0	13.7 $\pm$ 3.2
S850(PR22T)	MSHA-positive transductant	1.0	8	8.9 $\pm$ 2.9	16.2 $\pm$ 3.2
S850(PR16T)	MSHA-positive transductant	1.0	4	10.2 $\pm$ 1.9	15.6 $\pm$ 3.6

attach to HeLa cells to a reduced extent but attachment was inhibited by 0.1% (w/v) α -methyl-D-mannoside (Table 3). No agar-grown bacteria were internalized by HeLa cells even in the absence of α -methyl-D-mannoside; broth-grown bacteria invaded to the extent expected (Table 3).

MSHA-negative mutants. Non-fimbriate mutant strains TML(Y42) and TML(N12) did not elaborate the MSHA when grown in liquid medium but did show MRHA activity and normal motility. Such mutants adhered to HeLa cells and adhesion was not reduced in the presence of α -methyl-D-mannoside (Table 4). Strain TML(N15), an MSHA-positive revertant clone of strain TML(N12), was no more adhesive than strain TML(N12) (Table 4). Organisms of all four strains were internalized by the HeLa cells to similar extents (results not shown).

Table 6. *Haemagglutinating and adhesive properties of cultures grown at 18 °C and 37 °C*

Strain	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ s.d.)
<i>Growth at 37 °C</i>			
S850	0.0	16	16.1 $\pm$ 3.6
S2204	0.0	8	5.8 $\pm$ 1.6
TML	1.0	16	9.7 $\pm$ 3.3
W118	1.0	16	14.2 $\pm$ 3.3
NY	1.0	16	18.8 $\pm$ 4.4
PR	1.0	8	11.5 $\pm$ 4.0
<i>Growth at 18 °C</i>			
S850	0.0	<2	1.1 $\pm$ 1.7
S2204	0.0	16	6.4 $\pm$ 2.0
TML	0.15	16	7.7 $\pm$ 3.4
W118	0.65	<2	2.6 $\pm$ 2.0
NY	0.50	<2	1.5 $\pm$ 1.5
PR	0.30	<2	0.8 $\pm$ 1.5

Table 7. *Haemagglutinating and adhesive properties of MRHA-negative mutants*

Strain	Remarks	Relative MSHA activity	MRHA titre	Adhesion index for HeLa cells ($\pm$ s.d.)		Invasion index*
				α -MM absent	α -MM present	
TML	MRHA-positive wild-type	1.0	16	9.8 $\pm$ 2.8	10.6 $\pm$ 2.6	0.74 ^a
TML(16)	MRHA-negative mutant	0.8	<2	1.9 $\pm$ 2.4	0.6 $\pm$ 1.1	0.06 ^b
TML(54)	MRHA-negative mutant	1.0	<2	1.4 $\pm$ 1.6	1.2 $\pm$ 1.2	0.10 ^b
TML(72)	MRHA-negative mutant	1.0	<2	1.0 $\pm$ 1.8	0.9 $\pm$ 1.3	0.21 ^b
TML(161A)	MRHA-positive revertant	1.0	2	7.5 $\pm$ 2.6	9.6 $\pm$ 2.8	0.69 ^a

* Proportion of attached bacteria internalized (Jones *et al.*, 1981): *a*, significant proportion internalized ($P < 0.001$); *b*, proportion internalized insignificant ($P > 0.05$).

MSHA-positive transductants. The MSHA-positive transductants of strain S850 retained their ability to attach to HeLa cells (Table 5), but only when α -methyl-D-mannoside was present did the organisms attach to an extent similar to that of the recipient strain S850. In the absence of this carbohydrate, the transductants were only as adhesive as the donor strain PR (Table 5). Other MSHA-positive transductants of strain S850, derived with phage lysates of strain 18, behaved in a similar manner (results not shown). Similar proportions of attached organisms of all strains became intracellular within 3 h (results not shown).

MRHA-negative phenotypes. Bacteria of all six wild-type strains of *S. typhimurium* adhered to HeLa cells when grown in broth at 37 °C, whereas only strains TML and S2204 did so after growth at 18 °C (Table 6). Strains S2204 and TML were the only strains to show MRHA activity at the lower incubation temperature. Although MSHA production was also less at 18 °C than at 37 °C, the greatest reduction in MSHA activity was exhibited by cultures of the adhesive strain TML (Table 6).

MRHA-negative mutants. The three independently derived MRHA-negative mutants of strain TML [strains TML(16), TML(54) and TML(72)] failed to adhere to HeLa cells although normal motility and MSHA production was apparent (Table 7). After incubation with HeLa cell monolayers for 3 h in the standard invasion test, the total number of bacteria associated with the HeLa cells increased by a factor of about two, but little or no invasion of the HeLa cells occurred (Table 7).

The MRHA titres of cultures of the MRHA-positive revertant strain TML(161A) were seldom greater than 2 (Table 7). Nevertheless, the reappearance of this level of MRHA

activity in a culture coincided with the renewed ability of the bacteria to adhere to HeLa cells (Table 7). Conversely, the spontaneous loss of detectable MRHA activity (i.e. MRHA titre <2) from cultures of the more unstable revertants occurred concomitantly with the loss of the ability to adhere to HeLa cells.

DISCUSSION

The ability of non-fimbriate strains of the FIRN biogroup to attach to HeLa cells demonstrated clearly that type 1 fimbriae could not be responsible for the attachment of all *S. typhimurium* strains (summarized in Table 8, lines 1 and 2). Indeed, the mannose-resistant nature of the adhesive properties of bacteria of both fimbriate and non-fimbriate strains implicated an adhesive mechanism other than that provided by type 1 fimbriae which possess MSHA activity. Candidates for such a role are the MRHA activities which were detected in cultures of all six strains. These MRHA activities were superficially similar in that their detection was restricted to the microhaemagglutination test; they displayed similar agglutinating activities with erythrocytes of different species; and all were most active in the cold and none were inhibited by D-mannose. They appeared to differ only in respect to production at 18 °C, an observation that may indicate the existence of different types of MRHA activity. However, the two strains that produced MRHA at 18 °C are probably phylogenetically distinct, as is the one FIRN strain that failed to produce MRHA (Duguid *et al.*, 1975; Old & Duguid, 1979). It is more probable that the inhibition of MRHA production at 18 °C was strain dependent and perhaps due to difficulties in transportation of the MRHA to the microbial surface at this temperature. Such possibilities are indicated by studies on the temperature-dependent production of the K99 haemagglutinin (de Graff *et al.*, 1980).

The presence of MRHA activity was correlated with the ability to attach to HeLa cells in tests with broth cultures grown at 18 °C and 37 °C (Table 8, lines 6 and 7, 1 and 2), and in the analysis of MRHA-negative/MSHA-positive mutants and their MRHA-positive/MSHA-positive revertants (Table 8, line 5). The MRHA titre and the adhesion index, however, did not correlate. The haemagglutination test measured the MRHA content of the culture, whereas the adhesion test detected those cells capable of adhering to HeLa cells. If attachment to HeLa cells requires a certain amount of adhesin per bacterium, then all bacteria with adhesin in excess of this level would attach regardless of the haemagglutinin content of the whole culture.

The MSHA activity of fimbriate strains participated little, if at all, in the attachment of salmonellae to HeLa cells. Firstly, MRHA-negative/MSHA-positive mutants failed to adhere to any significant degree (Table 8, line 5). Secondly, MRHA-positive/MSHA-negative bacteria were as adhesive as the parent bacteria (Table 8, line 3). Thirdly, the fimbriate strain which produced the least amount of MSHA when grown at 18 °C was the only fimbriate strain to attach after growth at this temperature. Only in suspensions of agar-grown, non-motile bacteria that were constitutive for fimbriae production was mannose-sensitive attachment apparent (Table 8, line 9). It seems likely, however, that the mannose-sensitive and mannose-resistant forms of attachment require different conditions for their expression. For instance, motility is not essential for the attachment of non-motile *Shigella flexneri* to animal cells via type 1 fimbriae (Duguid & Gillies, 1957), whereas motility is necessary for the usual form of salmonella attachment to HeLa cells (Jones *et al.*, 1981). The present studies show this motility-dependent attachment to be mannose-resistant. Conversely, previous studies showed that agitation of the test mixture enhances the adhesive activity of type 1 fimbriae in *Escherichia coli* (Jones & Rutter, 1974), but agitation prevents the adhesion of salmonellae to HeLa cells (Jones *et al.*, 1981). Consequently, the contribution of type 1 fimbriae to attachment may be expected to be low under the static conditions of the HeLa cell test. Mannose-sensitive attachment, therefore, may be detected only occasionally when the mannose-resistant adhesin is absent or inactive or MSHA activity is enhanced.

Table 8. *Summary of results*

Results designated + indicate the property was present (motility >60%), — indicates no measurable activity existed (motility < 1%), and +/— indicates the property was strain dependent.

Phenotype	Representative strain(s)	Motility	MSHA activity	MRHA activity	Adhesion to HeLa cells		Invasion of HeLa cells
					α -MM* absent	α -MM* present	
<i>Growth in broth at 37 °C</i>							
1. FIRN	S850	+	—	+	+	+	+
2. Non-FIRN	TML	+	+	+	+	+	+
3. MSHA-negative	TML(Y42)	+	—	+	+	+	+
4. MSHA-positive	S850(PR22T)	+	+	+	+	+	+
5. MRHA-negative	TML(16)	+	+	—	—	—	—
<i>Growth in broth at 18 °C</i>							
6. MRHA-negative	PR/S850	+	+/—	—	—	—	—
7. MRHA-positive	TML/S2204	+	+/—	+	+	+	+
<i>Growth on agar at 37 °C</i>							
8. MSHA-negative	TML	—	—	+	—	—	—
9. MSHA-positive	TML(AU6)	—	+	+	+	—	—

* α -MM, α -Methyl-D-mannoside.

† Reduced adhesion.

The attachment of type 1 fimbriae is indicated by the observation that adhesion of fimbriate transductants was enhanced in the presence of D-mannose. This suggested that type 1 fimbriae bound to HeLa cells, but in so doing reduced attachment mediated by MRHA activity (Table 8, line 4). Inhibition by D-mannose of the binding of type 1 fimbriae could allow MRHA determinants to have greater access to the HeLa cell surface and hence cause increased bacterial attachment. Where MSHA type 1 fimbrial attachment is established it is likely that the distance between the animal cell surface and the bacterial cell is too great to allow MRHA-mediated attachment. This interpretation is consistent with a previous conclusion (Jones *et al.*, 1981) that the main *S. typhimurium* adhesin (i.e. MRHA) effectively promoted binding of bacteria to HeLa cells only when a small distance (about 15 nm) separated the two surfaces. The same explanation may be offered for the apparent inactivity of the MRHA of non-motile bacteria attached to HeLa cells in a mannose-sensitive manner (Table 8, line 9).

The opinion (reviewed by Kihlström, 1980) that rough cell-wall characteristics play an important role in the interaction of *S. typhimurium* with HeLa cells and the one presented here, that the MRHA of smooth bacteria is of importance, are reconcilable. Real differences may exist in some of the ways that rough and smooth salmonellae enter the initial phase of reversible attachment that precedes irreversible attachment or adhesion (Jones *et al.*, 1981). Initial contact by mutants with rough cell-walls, conceivably, could be caused by their adsorption to animal cells by virtue of the increased hydrophobic characteristics of their surface (Stendahl *et al.*, 1973; Van Oss, 1978). Smooth bacterial cells, in contrast, are known to achieve this initial contact through motility (Jones *et al.*, 1981). Once initial contact has been accomplished, however, the adhesion of both rough and smooth cells of *S. typhimurium* to HeLa cells may be similar and involve the MRHA.

The internalization of *S. typhimurium* by HeLa cells correlated only with mannose-resistant attachment of the bacteria to the HeLa cells. The form of attachment, therefore, appeared to play an important role in the internalization process. On the one hand, attachment may need to be specific in that only binding of the mannose-resistant adhesin to its HeLa cell receptor will affect internalization. On the other hand, internalization may depend on a minimal number of bonds being formed between the bacterium and HeLa cell

and/or the formation of bonds of a minimal strength (Griffin *et al.*, 1975; Capo *et al.*, 1978). In the latter case, it must be assumed that in the HeLa cell system these conditions are fulfilled only in MRHA-mediated adhesion. In other test systems in which the animal cell is a professional phagocyte, the binding of type 1 fimbriae is sufficient to enhance internalization (Silverblatt *et al.*, 1979). This apparent contradiction, therefore, may be the result of differences in specific ligand binding sites on the animal cells or due to the extent of type 1 fimbrial bonding of the bacteria to animal cells. There are few type 1 fimbrial binding sites, for example, on uroepithelial cells (Ørskov *et al.*, 1980) and some species of erythrocyte (Duguid & Old, 1980).

Salmonella typhimurium, therefore, produces two adhesins, each of which presumably has some specific and unique function. The precise nature of the putative mannose-resistant adhesin and how it may interact with animal cell receptors to facilitate bacterial entry into the host cell may be of importance to the understanding of these pathogens.

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