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Infection of HeLa Cells with *Salmonella typhimurium* 395 MS and MR10 Bacteria

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After interaction with HeLa cells cultured in vitro, the fraction of adhering extracellular and that of internalized smooth *Salmonella typhimurium* 395 MS and rough 395 MR10 have been determined by two different techniques. (i) By using the indirect fluorescent-antibody technique on unfixed and acetone-fixed HeLa cell preparations, intracellular bacteria were considered to become stained only after acetone fixation. (ii) Based on the assumption that gentamicin affects only extracellular bacteria, disintegration of the infected HeLa cells and viable count allowed the determination of internalized bacteria. Both techniques showed that MS as well as MR10 bacteria gained intracellular access, the fraction of MR10 cells doing so being much greater. The net increase in the intracellular bacterial population was small within 3 h of incubation.

Many microorganisms gain access to the host at mucosal surfaces. Some, e.g., *Vibrio cholerae*, exert their effect via enterotoxin production and usually do not pass the mammalian cell membrane (19). Others, e.g., *Salmonella typhimurium*, are more invasive and predominantly exert their effect after passage through the mucosal barrier (19), although enterotoxic factors have been described for some strains (13, 18).

Attachment and ingestion of *S. typhimurium* by polymorphonuclear leukocytes is facilitated after S → R mutation (2, 22) and after opsonization with immunoglobulin G (24). Both processes increase the liability to hydrophobic and charge interaction as shown by partition in aqueous two-phase systems (23-25; K.-E. Magnusson, O. Stendahl, C. Tagesson, L. Edebo, and G. Johansson, Acta Pathol. Microbiol. Scand., in press) and measurements of the contact angle of a drop of saline on a layer of cells (2). A similar facilitation of the association of *S. typhimurium* R as compared with S bacteria has been shown with HeLa cells (12). In these experiments, however, it was not demonstrated whether the associated bacteria gained intracellular access. A variety of techniques have been devised to assay the association of bacteria with mammalian cells (26), but few differentiate between attachment and internalization (1, 3, 8). The present work employs two different techniques with the aim of distinguishing quantitatively the attached from the internalized bacteria when *S. typhimurium* 395 MS and MR10 infect HeLa cells.

MATERIALS AND METHODS

Bacterial strains. The smooth, mouse-virulent strain *S. typhimurium* 395 MS and its R-mutant 395 MR10 (chemotype Rd) derived from it have been characterized earlier (5, 11, 14).

Cultivation of bacteria. All strains were kept at 4°C on agar slants before use. The bacteria were inoculated into 15 ml of glucose broth and incubated at 37°C for 18 h. The bacteria were harvested by centrifugation (1,100 × *g*, 15 min), washed twice in phosphate-buffered saline solution (PBS; pH 7.3), and suspended to 4.0 × 10^7 bacteria/ml (estimated with a Turner spectrophotometer at 650 nm) in Earle balanced salt solution (EBSS; pH 7.3; Flow Laboratories, Irvine, Scotland).

Preparation of antigen and immunization procedure. The bacteria were grown for 18 h in a synthetic medium containing (per liter of medium): K_2HPO_4, 7.5 g; $NaH_2PO_4 \cdot 2H_2O$, 3.0 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; glucose, 10.0 g; and a standard trace element solution. After centrifugation, the bacteria were resuspended in PBS, and the concentration was determined with a Turner spectrophotometer at 650 nm. The bacteria were killed at 56°C for 1 h and tested for sterility. White rabbits were injected intravenously with 0.2-mg amounts of the heated bacteria three times a week for 9 weeks (4).

Mammalian cell culture and its interaction with bacteria. The HeLa cell line (ATCC strain CCL 2, human serum, research grade) was obtained from Flow Laboratories, Irvine, Scotland. Specimens of the cells were kept in liquid nitrogen and initiated at intervals not longer than 3 months (20 passages). The cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, and 100 IU of streptomycin per ml. Cells were maintained as monolayers in glass or plastic tissue culture bottles in a humidified incuba-

tor (ASSAB, Sundbyberg, Sweden) with an atmosphere of 5% CO_2 and 95% air at 37°C. The monolayer was treated with 0.25% trypsin to detach the cells, and the detached cells were used to prepare monolayers. Monolayers to be used for immunofluorescence studies were prepared on cover slips (18 by 18 mm) that had been attached with petrolatum to the bottom of a tissue culture petri dish (50 by 13 mm). HeLa cells for disintegration were cultivated in petri dishes without cover slips. The petri dishes were seeded with 2 ml of HeLa cell suspension and 1 ml of fresh medium. The dishes were incubated for approximately 72 h in the humidified incubator with changes of medium every 24 h, the last medium containing neither penicillin nor streptomycin. The cells were regularly tested for mycoplasma contamination by staining with orcein (7). All cell culture material was purchased from Flow Laboratories, Irvine, Scotland.

After incubation for 72 h, the medium was poured off from the petri dishes, the bottoms were rinsed three times with 37°C PBS, 3 ml of bacterial suspension was added, and the petri dishes were incubated again in the humidified incubator.

Discrimination between extracellular and intracellular bacteria with fixation and immunofluorescence. After 3 h of incubation with bacteria, the petri dishes with HeLa cells were rinsed three times with PBS and incubated for 30 min at 20°C with antiserum diluted 1:5 with PBS. MS bacteria were incubated with anti-MS serum, and MR10 bacteria were incubated with anti-MR10 serum. The dishes were then rinsed with PBS, and 5% fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit 7S immunoglobulin (Hyland Laboratories, Inc., Div., Travenol Laboratories Inc., Costa Mesa, Calif.) was added; the cells were then incubated for 30 min at 20°C, rinsed with PBS, and mounted. Where indicated, the monolayers were fixed in acetone for 10 min before the antiserum against the bacteria was added. Fixation was used because it is considered to facilitate the passage of antibodies through the cell membrane (20) and consequently allows the quantitation of the total number of HeLa cell-associated bacteria. With unfixed HeLa cells only the adhering extracellular bacteria are observed because of the inability of the antibodies to penetrate the cell membrane (3, 29). The following controls were included: (i) noninfected cells plus specific antibody plus FITC-labeled antiglobulin; (ii) infected cells plus normal rabbit serum plus FITC-labeled antiglobulin; and (iii) infected and noninfected cells plus FITC-labeled antiglobulin (20).

Specimens were examined with epifluorescence in a Zeiss Universal microscope with two exciter filters (KP500) and barrier filters (44 and 50) and at a total magnification of 1,250. A total of 100 HeLa cells were randomly selected on each cover slip and ordered into six groups, containing 0, 1, 2, 3 to 5, 6 to 10, and more than 10 fluorescent bacteria/HeLa cell, respectively. The percentage of intracellular bacteria was estimated as: $[(n_{fixed} - n_{unfixed})/n_{fixed}] \times 100$, where n is the number of bacteria associated with 100 HeLa cells in the fixed and unfixed preparations, respectively.

Determination of extracellular and intracellular bacteria by killing extracellular bacteria with gentamicin and recovery of intracellular bacteria after disintegration of the HeLa cells. Petri dishes with monolayers of HeLa cells were incubated with bacteria as above. The monolayers were then rinsed three times in 37°C PBS and detached from the petri dishes with 0.25% trypsin and a rubber policeman. The HeLa cells were suspended in 3 ml of EBSS plus 24 ml of PBS and disintegrated with a modified LoX-press (27) at an extrusion pressure of 5.3×10^6 to 6.1×10^6 Pa. This procedure disrupted nearly all of the HeLa cells without killing the bacteria (12). Samples were removed from the homogenate for viable counts which were taken to represent the number of adhering extracellular plus intracellular bacteria (n_{total}). To eliminate the extracellular bacteria, the bacterial suspension was poured off the petri dishes, the dishes were rinsed three times in 37°C PBS, and different concentrations of gentamicin in EBSS were added; the cells were incubated further before disintegration and viable counts were performed. These viable counts were considered to represent intracellular bacteria (n_{intra}). The percentage of intracellular bacteria was calculated as $(n_{intra}/n_{total}) \times 100$. The n_{total} used was taken before the addition of gentamicin, thus being the same for the different times of incubation with gentamicin.

RESULTS

Determination of the fraction of intracellular bacteria in HeLa cells by immunofluorescence. *S. typhimurium* 395 MR10 became associated with HeLa cells to a greater extent than did MS, as observed on acetone-fixed as well as on unfixed preparations (Fig. 1A and B). For MS about the same number of bacteria were associated with fixed and unfixed preparations; i.e., 40 to 45% of the HeLa cells showed association with at least one bacterium (Fig. 1A). For MR10 about 98% of fixed HeLa cells showed interaction with bacteria, and 74% of these showed interaction with more than 10 bacteria. The corresponding figures for unfixed HeLa cells were 76 and 30%, respectively (Fig. 1B). The data further show that several HeLa cells that without fixation showed only 0 to 2 bacteria associated revealed more than 10 bacteria after fixation (Fig. 1B). Although the present technique does not allow an accurate quantitation of the number of cell-associated bacteria, about 50% of the number of associated MR10 seemed to be intracellular, whereas the number of HeLa cell-associated MS was reduced (−17%) after acetone fixation, indicating that few, if any, MS became intracellular.

All three groups of control experiments were performed on both fixed and unfixed HeLa cell preparations. More than 90% of the HeLa cells did not show any fluorescence, which is similar to that of the bacteria.

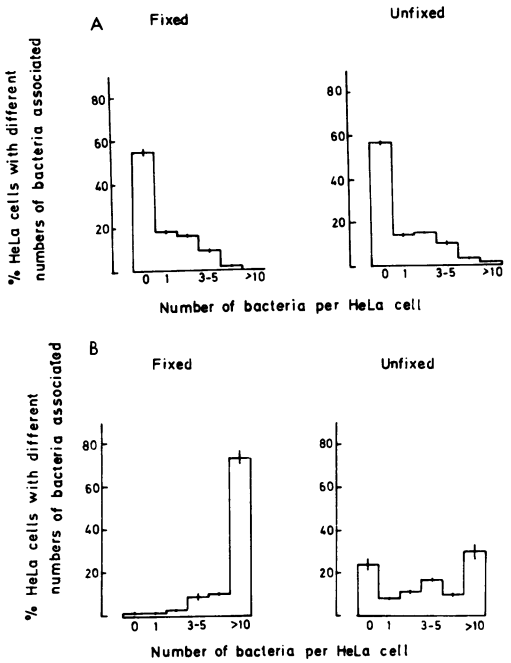

FIG. 1. *Association of S. typhimurium 395 MS (A) and 395 MR10 (B) with fixed and unfixed HeLa cells. Vertical bars show the standard error of the mean for 11 to 12 experiments.*

Effect of gentamicin on *S. typhimurium* 395 MS and MR10. The minimum bactericidal concentration of gentamicin on *S. typhimurium* 395 MS and MR10 grown in nutrient broth was 2.5 μg/ml.

When *S. typhimurium* 395 MS and MR10 were added to petri dishes without HeLa cells and incubated for 1.5 h, some of the bacteria adhered to the petri dishes. The number of attached bacteria was determined by viable counts when the petri dishes had been washed three times in 37°C PBS and the bacteria had been detached from the petri dishes with 0.25% trypsin and a rubber policeman. When the petri dishes with the attached bacteria were treated with different concentrations of gentamicin for 20 min or 1.5 h before viable counts, the ability of gentamicin to kill attached bacteria was determined. A 10-μg amount of gentamicin per ml killed 69.5% of the adherent MR10 during 20 min and 99.7% during 1.5 h. The corresponding figures for MS were 91.4 and 99.7%, respectively. By increasing the concentration of gentamicin to 40 μg/ml, 99.3% MR10 and 99.5% MS were killed in 20 min. A 100-μg amount of gentamicin per ml gave approximately the same values as did 40 μg/ml (Table I). Therefore, 10 μg of gentamicin per ml was incubated for ≥1.5 h, and 40 and 100 μg/ml were incubated for ≥20 min to determine the intracellular fractions of MS and MR10 associated with HeLa cells.

Determination of the fraction of intracellular bacteria in HeLa cells after disintegration. The HeLa cells infected with *S. typhimurium* 395 MS or MR10 were disintegrated, and viable counts on the homogenates were performed to determine the total number of associated bacteria. In some experiments the infected HeLa cells were treated with gentamicin before disintegration to count only the intracellular ones that yielded significantly more MR10 than MS (Tables 2 through 4). By increasing the concentration of gentamicin from 10 to 40 and 100 μg/ml and incubating for 1.5 h, the recovery of MR10 was decreased from 26.9 to 15.7 and 17.9%, respectively ($P < 0.05$, Student's t test [Table 3]). A similar decrease of viable intracellular bacteria at increased concentrations of gentamicin was seen after 3.5 h with MR10 (Table 3), as well as with MS bacteria (Table 2). By increasing the time of incubation with gentamicin (40 μg/ml) from 1.5 to 3.5 h, the recovery of MR10 bacteria increased from 15.7 to 28.5% ($P < 0.10$, Student's t test [Table 3]). Only a small effect could be seen for MS bacte-

TABLE 1. *Inactivation of S. typhimurium 395 MS and 395 MR10 adherent to plastic petri dishes by different concentrations of gentamicin*

Concn of gentamicin (μg/ml)	Incubation time with gentamicin (min)	Killed bacteria (%)	
		MS	MR10
10	20	91.4	69.5
10	90	99.7	99.7
40	20	99.5	99.3
100	20	99.7	99.3
100	90	100	100

TABLE 2. *Percent intracellular S. typhimurium 395 MS in HeLa cells[a] estimated after incubation with different concentrations of gentamicin for different times*

Time with gentamicin	Intracellular *S. typhimurium* 395 MS (%) at gentamicin concn (μg/ml) of:		
	10	40	100
20 min	ND[b]	1.7 (1)[c]	2.8 (2)
1.5 h	7.2 ± 1.3[d] (6)	3.6 (1)	5.1 ± 0.4 (4)
3.5 h	7.7 ± 1.2 (5)	4.5 (1)	5.3 (2)

[a] Monolayers of HeLa cells were preincubated for 1.5 h before the addition of gentamicin.
[b] ND, Not done.
[c] Numbers in parentheses indicate the number of experiments.
[d] Mean ± standard error of the mean.

TABLE 3. *Percent intracellular S. typhimurium 395 MR10 in HeLa cells[a] estimated after incubation with different concentrations of gentamicin for different times*

Time with gentamicin	Intracellular *S. typhimurium* 395 MR10 (%) at gentamicin concn (μg/ml) of:		
	10	40	100
20 min	ND[b]	17.7 (2)[c]	21.1 ± 0.3[d] (4)
1.5 h	26.9 ± 1.6 (4)	15.7 ± 1.4 (4)	17.9 ± 1.9 (3)
3.5 h	41.1 ± 7.9 (5)	28.5 ± 4.2 (5)	18.7 ± 2.1 (3)

[a] Monolayers of HeLa cells were preincubated for 1.5 h before the addition of gentamicin.
[b] ND, Not done.
[c] Numbers in parentheses indicate the number of experiments.
[d] Mean ± standard error of the mean.

TABLE 4. *Percent intracellular S. typhimurium 395 MS and 395 MR10 in HeLa cells[a]*

Preincubation time (h)	Intracellular *S. typhimurium* (%)	
	MS	MR10
1.5	1.7 (1)[b]	17.7 (2)
3	4.8 ± 1.1[c] (4)	27.3 ± 1.2 (4)

[a] Monolayers of HeLa cells were preincubated for different periods before incubation with gentamicin (40 μg/ml for 20 min), disintegration, and viable counts.
[b] Numbers in parentheses indicate the number of experiments.
[c] Mean ± standard error of the mean.

ria (Table 2). When HeLa cells were preincubated for 3 instead of 1.5 h before the addition of gentamicin, the recovery of MR10 was increased from 17.7 to 27.3% ($P < 0.10$, Student's t test [Table 4]). The corresponding figures for MS were 1.7 and 4.8%, respectively (Table 4).

DISCUSSION

To elucidate the pathogenesis of enteric bacterial infections, it is of great importance to distinguish between adhering and intracellular bacteria. No simple method exists for the distinction between extra- and intracellular bacteria. Antibodies do not penetrate the cell membrane of intact mammalian cells (3, 29), whereas pretreatment with acetone, for example, allows penetration and binding to intracellularly localized bacteria (20). By using the indirect immunofluorescence technique, this effect can be used to estimate the proportion of intracellular bacteria. With smooth *S. typhimurium* 395 MS this approach showed that almost all associated bacteria were extracellular, whereas with the rough MR10 about 50% were intracellular. It should be observed that this technique does not distinguish living from killed bacteria and has the drawback that exact quantitation is difficult. Therefore, the alternative technique with gentamicin was applied under similar conditions.

By treating infected HeLa cell monolayers with different concentrations of gentamicin for different lengths of time, the proportion of surviving bacteria was always lower for MS than for MR10 compared with monolayers not treated with gentamicin (Tables 2 through 4). Several authors claim that intracellular bacteria are protected from the bactericidal actions of non-lipid-soluble antibiotics, e.g., gentamicin (15, 16). The surviving bacterial fraction after gentamicin treatment is therefore considered to be intracellular. The mechanism for this protection is still obscure, and several hypotheses have been suggested: (i) a barrier at the cell boundary may impede the transfer of the drug into the cell, (ii) environmental factors may interfere with antibiotic activity intracellularly, or (iii) intracellular pathogens may be in a metabolic state that render them "drug indifferent" (21).

By increasing the concentration of gentamicin from 10 to 40 and 100 μg/ml with a constant time of incubation, fewer surviving MS and MR10 were recovered (Tables 2 and 3). This occurred despite the fact that 10 μg of gentamicin per ml was sufficient to kill more than 99% MS and MR10 adhering to tissue culture petri dishes in the absence of HeLa cells if incubated for 1.5 h or more, and 10 μg/ml was well above the minimum bactericidal concentration value for gentamicin on these bacteria. This showed that the fraction of surviving bacteria was dependent on the concentration of gentamicin. The reason for this might have been an inability of 10 μg of gentamicin per ml to gain access to all cell membrane-attached bacteria or that higher concentrations of gentamicin might have affected intracellular bacteria.

When the time of incubation with gentamicin (10 and 40 μg/ml) was increased from 1.5 to 3.5 h, the surviving MR10 fraction increased significantly, but only a minimal increase was seen for MS (Tables 2 and 3). This might have depended on an increased internalization of bacteria or intracellular multiplication. Since very few extracellular bacteria survived and therefore could not significantly contribute to the increased number of intracellular bacteria, the latter explanation seems more likely. Furthermore, van Oss et al. (30) have stated that gentamicin (10 μg/ml) alters the surface properties of *Escherichia coli* and human neutrophils, as assessed by contact angle measurements, so as to increase the phagocytosis. However, in the

present communication gentamicin was added after the interaction between bacteria and HeLa cells. Therefore, the attachment of bacteria to HeLa cells was not affected. Whether the ingestion phase is influenced by gentamicin remains to be proved. No increase in the intracellular fraction was seen when the time of incubation with gentamicin was increased from 20 min to 1.5 h for MR10. It is thus reasonable to consider the intracellular division rate to be reduced, at least for MR10, as the division rate of *S. typhimurium* in the spleen of mice has been shown to be only 5 to 10% of the maximum observed in vitro (17). When the concentration of gentamicin was increased to 100 μg/ml, no increase in intracellular bacteria occurred between 1.5 and 3.5 h, which indicates that at this concentration enough gentamicin occurred intracellularly to affect the viability of the bacteria. The constant number of bacteria in cultures with 100 μg/ml might be a result of balancing growth and killing.

When the preincubation (without gentamicin) with bacteria and HeLa cells was prolonged from 1.5 to 3 h, the surviving fraction increased for both MS and MR10 (Table 4). Thus, the association of bacteria with HeLa cells (12) as well as the intracellular localization increased with time. Gerber and Watkins (9) pointed out that 10 μg of streptomycin per ml depressed intracellular multiplication of *Shigellae* in Henle 407 intestinal epithelial cells. Instead, they controlled extracellular multiplication by washing the infected monolayer. A similar depression was observed with gentamicin at 100 μg/ml (Table 3), but might also have existed at 40 μg/ml, since preincubation without gentamicin for 3 h plus 20 min with gentamicin yielded a surviving fraction of 27.3% (Table 4), whereas preincubation without gentamicin for 1.5 h plus 1.5 h with gentamicin yielded only 15.7% (Table 3). Before adopting the gentamicin technique, we tried to eradicate extracellular and cell membrane-attached bacteria by repeated washings, but the bacterial content in the last monolayer wash fluid was difficult to decrease sufficiently so as not to influence the number of bacteria in the homogenate. Furthermore, the bacteria tended to stick to the plastic petri dishes even in the absence of HeLa cells. We therefore chose to eradicate cell membrane-attached bacteria by treatment with an antibiotic with weak intracellular activity. Gentamicin seemed to fulfill this criterion (15) and is not considered to influence cellular physiology (6) in the concentrations used.

A direct numerical comparison between the two methods used may not be appropriate because the threshold for placing the bacteria inside or outside the cell membrane might be different for the two methods as it is for different concentrations and times of incubation of gentamicin. However, the experiments clearly show that both MS and MR10 infect HeLa cells; MR10 cells always do so more efficiently. This might seem to be a paradox considering the much higher virulence of MS than that of MR10 (5). However, it is in good agreement with the hypothesis that the high virulence of MS is less dependent on a particular aggressiveness toward cells from susceptible hosts than on its capacity to evade and survive the host defense (Magnusson et al., Acta Pathol. Microbiol. Scand., in press). The noncharged and highly hydrophilic character of the MS bacteria (Magnusson et al., Acta Pathol. Microbiol. Scand., in press) is likely to determine the low tendency to associate with phagocytes (22) as well as with HeLa cells. Futhermore, Giannella et al. (10) state that there was no correlation between the ability of *S. typhimurium* to invade rabbit ileum and fluid secretion. Only invasive strains caused fluid exsorption, although some invaded the mucosa without causing fluid secretion. This may also help explain the paradox that a nonvirulent R-mutant is more invasive than the parent S-strain. Furthermore, all invasive strains caused acute mucosal inflammation. Since R-mutants are more liable than the S-strain to undergo phagocytosis (22), it is likely that the R-mutants are more inhibited by an acute inflammatory reaction, whereas S-strains may survive and disseminate across the intestinal wall (28). Substances of lysosomal or nonlysosomal origin from the epithelial cells may also contribute to the bactericidal defense system (C. Tagesson, personal communication) and together with bacterial chemotactic factors, for example, induce an acute inflammatory reaction.

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