INFECTION AND IMMUNITY, Dec. 1993, p. 5013–5020
0019-9567/93/125013-08$02.00/0

Vol. 61, No. 12

Effects of Multiplicity of Infection, Bacterial Protein Synthesis, and Growth Phase on Adhesion to and Invasion of Human Cell Lines by *Salmonella typhimurium*

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Received 27 January 1993/Returned for modification 22 March 1993/Accepted 6 September 1993

Monolayers of intestine 407 (Int-407) cells were infected with the virulent *Salmonella typhimurium* strain C52, and the adhesion to and invasion of these cells were studied. The effects of the multiplicity of infection and growth phase of the bacteria (logarithmic versus stationary) on the interaction with eukaryotic cells were investigated. In contrast to other reports, we found no differences in the adhesive and invasive capacities of bacteria derived from logarithmic- or stationary-phase cultures. Invasion by *S. typhimurium* required bacterial protein synthesis and live Int-407 cells. Bacteria adhered equally well to dead or live Int-407 cells, which indicates that adhesion does not require metabolically active cells. Adhesion of *S. typhimurium* followed saturation kinetics, with a maximum of 10 adhesive bacteria per cell. This indicates that there is a limited number of bacterial adhesion sites (receptors) available on the surface of the host cell. Killed and live bacteria adhered equally well and competed with each other for cellular adhesion sites. This and adhesion experiments performed in the presence of chloramphenicol showed that bacterial protein synthesis is not required for adhesion. The general validity of the results obtained with *S. typhimurium* C52 was confirmed by comparing the invasion and adhesion data with those of the frequently used SL1344 and SR11 strains. In addition, we assayed the adhesion and invasion of *S. typhimurium* C52, SL1344, and SR11 and 27 *S. typhimurium* field isolates with Int-407, HeLa, and HEp-2 cells.

Salmonellae are pathogens in both humans and animals. They can cause disease ranging from gastroenteritis to typhoid fever, depending on the serotype of the bacterium and the infected host. Salmonellae possess several virulence determinants that interact with the host in a complex way (6). Pathogenic *Salmonella* species are able to enter (invade) the epithelial cells of the intestine (11, 20), and a large number of distinct genetic loci involved in *Salmonella* invasion has been reported (1, 4, 6, 8, 11–13, 15, 19, 21, 23, 27). Mutations in some of these genes reduce the capacity to penetrate intestinal cells without affecting adhesion (11, 15). This demonstrates that invasion is a two-stage process consisting of adhesion followed by penetration. However, most of the commonly used invasion assays do not discriminate between adhesion and penetration.

Invasion of epithelial cells by salmonellae has been studied extensively (1, 4–9, 11, 14, 15, 18, 19, 22, 23, 27, 28). Although these studies have increased our insight into the invasion mechanisms used by salmonellae, some contradictory observations have been reported and essential questions remain unanswered. The intracellular environment was shown to induce the synthesis of specific proteins (3). Some studies claim that invasion of epithelial cells by *Salmonella* spp. is a slow process that requires adaptive de novo protein synthesis which is induced by the contact of the bacterium with cells (7). Other studies (9) claim that invasion occurs instantaneously and without an apparent need for de novo protein synthesis (5). It was also reported that *Salmonella* spp. derived from anaerobically grown cultures are more invasive than bacteria from aerobic cultures, and bacteria from stationary cultures were shown to be less invasive than logarithmically growing bacteria (5, 19, 28). The 2-h lag time preceding invasion, reported by Finlay et al. (7), was originally interpreted as an indication for the necessity of the de novo production of bacterial proteins, but it is now attributed to the use of bacteria in a state equivalent to a stationary-phase culture (9).

Invasion of *Salmonella* spp. is assumed to be receptor mediated, but nothing about the nature of the receptor is known (4, 8, 11, 15). The epidermal growth factor receptor is involved in the invasion of cultured cells by *Salmonella typhimurium* (14), but it is unknown whether the bacterium directly interacts with this receptor.

We address some of the questions and controversies mentioned above. In this article, we define invasion as a two-stage process consisting of bacterial attachment (adhesion) to the host cell followed by penetration into the cell. We separated adhesion from penetration by using fixated Int-407 cells to determine the adhesion (7) and live Int-407 cells to measure invasion. We determined the effect of the number of bacteria added per cell and the growth stage of the bacterium on adhesion and invasion. We also examined the need for de novo protein synthesis of the bacterium in both the adhesion step and the penetration step of the invasion process.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* SR11 (16) and SL1433 (17) were obtained from R. Curtiss III (Washington University, St. Louis, Mo.). Strain C52 (24) is derived from the

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wild-type strain C5 (10, 25) and was obtained from L. Norel (Institute Pasteur, Paris, France). Strain C52 was chosen for performing detailed studies because it is not related to the frequently used SL1344 and SR11 strains. All three strains mentioned above are highly invasive in in vitro invasion assays and are fully virulent for mice. The nonadhesive, noninvasive *Escherichia coli* strain HB101 (2) was used as a negative control in all experiments. In some experiments, a clinical isolate of *Yersinia enterocolitica* (strain HK59; kindly donated by J. Dankert, AMC, Amsterdam, The Netherlands) was used as a control. In addition, we used 27 *S. typhimurium* field isolates from our own collection. These field strains (10 human, 9 bovine, 4 avian, 2 porcine, and 2 equine isolates) were isolated in the Netherlands from individuals who showed clinical signs of salmonellosis. All strains used in this study were sensitive to chloramphenicol (40 μg/ml; Sigma Chemical Co., St. Louis, Mo.), spectinomycin (300 μg/ml; Sigma), rifampin (120 μg/ml; Sigma), and colistin (polymyxin E, 150 μg/ml; AUV, Cuijk, The Netherlands). Bacterial strains were stored at −70°C in Luria-Bertani medium (LB) (26) supplemented with 20% glycerol.

Cell cultures. The cell lines used in this study, Int-407 (derived from human embryonic jejunum and ileum), HeLa (human cervical carcinoma), and HEp-2 (human larynx carcinoma) were obtained from Flow Laboratories, Inc. (McLean, Va.). Cells were cultured in disposable dishes and flasks from Costar (Cambridge, Mass.). All other media and reagents for tissue culture were obtained from GIBCO Laboratories (Life Technologies, Inc., Chagrin Falls, Ohio). Cells were routinely grown and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated (30 min, 56°C) fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml, in an atmosphere of 95% air and 5% CO_2. For invasion and adhesion assays, 10^5 cells were seeded per 35-mm tissue culture dish and incubated to confluency (approximately 10^6 cells per dish).

Growth and preparation of bacteria. Stationary-phase cultures of bacteria (≈2 × 10^9 CFU/ml) were prepared by inoculating LB with bacteria (≈10^3 CFU/ml) from frozen glycerol stocks and incubating them with vigorous shaking for 18 h at 37°C. Logarithmic-phase cultures (≈5 × 10^7 CFU/ml) were obtained by diluting a sample of the stationary-phase cultures 100-fold in prewarmed LB (37°C) and then incubating it for 2 h at 37°C. Bacterial cultures were collected by centrifugation (5 min at 3,000 × *g*) and suspended in prewarmed (37°C) DMEM without supplements. From this suspension, dilutions of 5 × 10^4 to 5 × 10^7 bacteria per ml were prepared, resulting in a multiplicity of infection (MOI) of 0.1 to 100 bacteria per cell in 2 ml of inoculum. To test the effect of the inhibition of bacterial protein synthesis, chloramphenicol (40 μg/ml) was added to the bacterial culture 30 min prior to the centrifugation step and to the DMEM used to prepare the bacterial inoculum. In some experiments, killed bacteria were added to the inoculum. These were prepared by suspending bacterial pellets (prepared as described above) in cold 2% glutaraldehyde (Merck-Schuchardt, Hohenbrunn, Germany) in phosphate-buffered saline (PBS) (26) and then incubating them for 1 h at 4°C. After centrifugation (5 min at 3,000 × *g*), the glutaraldehyde-PBS was removed and the bacteria were resuspended in DMEM and incubated at 4°C for 30 min. After two additional 30-min DMEM washes, the bacteria were resuspended and diluted in prewarmed DMEM as described above.

Invasion assay. Confluent monolayers of Int-407 cells were rinsed once with 4 ml of prewarmed DMEM and subsequently incubated with 2 ml of prewarmed DMEM for 30 min in a CO_2 incubator to remove residual antibiotics and serum components. The bacterial inoculum (2 ml per dish in DMEM) was added (time zero [t = 0]), and the infected cells were incubated at 37°C for 5 to 180 min (t = 5 to 180) in an atmosphere of 95% air–5% CO_2. Cells were washed five times with 4 ml of PBS to remove the nonadherent bacteria and subsequently incubated for an additional 90 min with fresh DMEM containing 150 μg of colistin per ml. Colistin was used rather than the commonly used gentamicin since *S. typhimurium* C52 and most field isolates are resistant to gentamicin. To validate the use of colistin for killing extracellular bacteria, we assayed the invasion of the frequently used *S. typhimurium* SR11 and SL1344 strains with 150 μg of either colistin or gentamicin per ml. After this 90-min incubation, the monolayers were washed three times with 4 ml of PBS to remove the antibiotic. Subsequently, the cells were overlaid with PBS containing 1% (vol/vol) Triton X-100 (1 ml per dish) and incubated for 5 min at room temperature to release the bacteria from the cells. The bacteria were resuspended in this solution, diluted, and plated on LB plates to determine the number of CFU. Background levels for invasion were determined by performing the assay described above with the noninvasive *E. coli* strain HB101 and with *E. coli* HB101 and *S. typhimurium* on dishes without cells. Bacterial growth occurring during the experiments was monitored by determination of the CFU of inocula added to dishes without cells (referred to as the total number of bacteria).

Adhesion assays. To determine the number of bacteria that adhered to glutaraldehyde-fixated cells, confluent monolayers of Int-407 cells (in 35-mm dishes) were washed twice with cold (4°C) PBS and incubated in 2% glutaraldehyde in PBS (2 ml per dish) at 4°C for 1 h. The glutaraldehyde solution was removed, and the dishes were rinsed once with 4 ml of cold PBS and then incubated at 4°C for 30 min with 4 ml of PBS per dish. This 30-min wash was repeated three times. Cells were then rinsed with 4 ml of prewarmed DMEM. The bacterial inocula (2 ml per dish) were added to the cells as described above for the invasion assays. Subsequently, the inoculum was removed and the cells were washed five times with 4 ml of PBS to remove the nonadherent bacteria. Bacteria were released by lysis of the cells. This and the subsequent determination of CFU were performed as described for the invasion assay.

We also determined the adhesion to live, nonfixated Int-407 cells. To prevent penetration of the cells (see Results), chloramphenicol (40 μg/ml) was added to the bacterial culture 30 min prior to the centrifugation step and to the DMEM used to prepare the bacterial inoculum. All further steps were performed as described above.

RESULTS

Invasion. Invasion of Int-407 cells was tested at different MOIs with logarithmic- and stationary-phase cultures of *S. typhimurium* C52. The number of intracellular bacteria increased with prolonged incubation times and higher MOIs (Fig. 1). The maximum number of intracellular bacteria (t = 180 min) ranged from approximately 0.05 (MOI = 0.1) to 2 (MOI = 100) bacteria per cell. When added at equal MOIs, stationary- and logarithmic-phase bacteria were equally invasive.

To test whether invasion of Int-407 cells by *S. typhimurium* C52 requires bacterial protein synthesis, we also tested

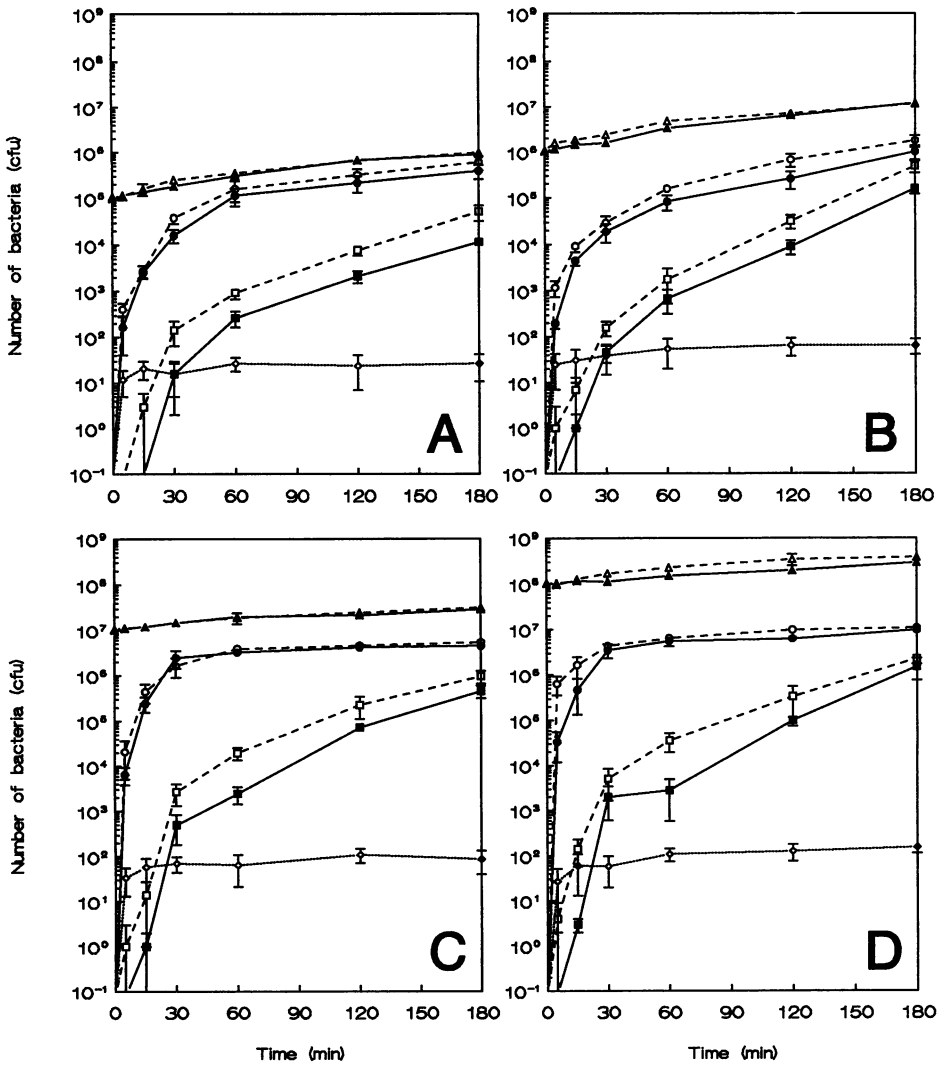

FIG. 1. Effects of the MOI on adhesion to and invasion of Int-407 cells by *S. typhimurium* C52. Symbols: ▲ and △, total number of bacteria as an indication of bacterial growth (see Materials and Methods); ● and ○, adhesive bacteria; ■ and □, colistin-resistant (intracellular or invasive) bacteria; △, ○, and □, bacteria in mid-log phase; ▲, ●, and ■, bacteria in stationary phase. As a negative control, the adhesion of logarithmically grown *E. coli* HB101 cells was determined (◇). The values of the additional adhesion controls (HB101 and C52 adhesion to culture dishes without cells) were always below these values (not shown). The same controls were also used in the invasion experiments, but no intracellular bacteria were found (not shown). Int-407 cells were infected with bacteria at an MOI of 0.1 (A), 1 (B), 10 (C), or 100 (D). The data represent the statistical means of three experiments. Error bars (representing the sample standard error) were omitted if these bars were smaller than the symbols.

invasion in the presence of the bacterial protein synthesis inhibitor chloramphenicol. Chloramphenicol reduced the invasion of both logarithmic- and stationary-phase cultures to background levels (Fig. 2A and B). As a control, the invasion by *Y. enterocolitica* was tested. The invasion of *Y. enterocolitica* was not significantly affected by the addition of chloramphenicol (Fig. 2C and D).

To study the role of bacterial protein synthesis in invasion in more detail, chloramphenicol was added at different times during the invasion process. Addition before the bacteria were added to the cells and up to 15 min after the addition of the bacteria to the cells completely prevented the bacteria from entering the cells (Fig. 3). If chloramphenicol was added at a *t* of 30 min or thereafter, it immediately stopped further invasion (Fig. 3). Similar results were obtained when another protein synthesis inhibitor (300 μg of spectinomycin per ml) or an inhibitor of RNA synthesis (120 μg of rifampin per ml) was used (data not shown).

Adhesion. The adhesion of logarithmic- and stationary-phase cultures of *S. typhimurium* tested at different MOIs is shown in Fig. 1. Bacteria always adhered instantly to the cells. The adhesion rate depends on the MOI, but there was a maximum of approximately 10 bound bacteria per cell.

There were no striking differences between the adhesion rates of stationary- and logarithmic-phase bacteria. However, as with the invasion experiments, we always observed slightly more adhesive bacteria with logarithmic-phase than with stationary-phase cultures. This difference can be explained by the presence of dead bacteria in the stationary-phase culture. Dead bacteria may compete for adhesion sites, while their presence is not reflected in the number of CFU determined. This was confirmed by testing the adhe-

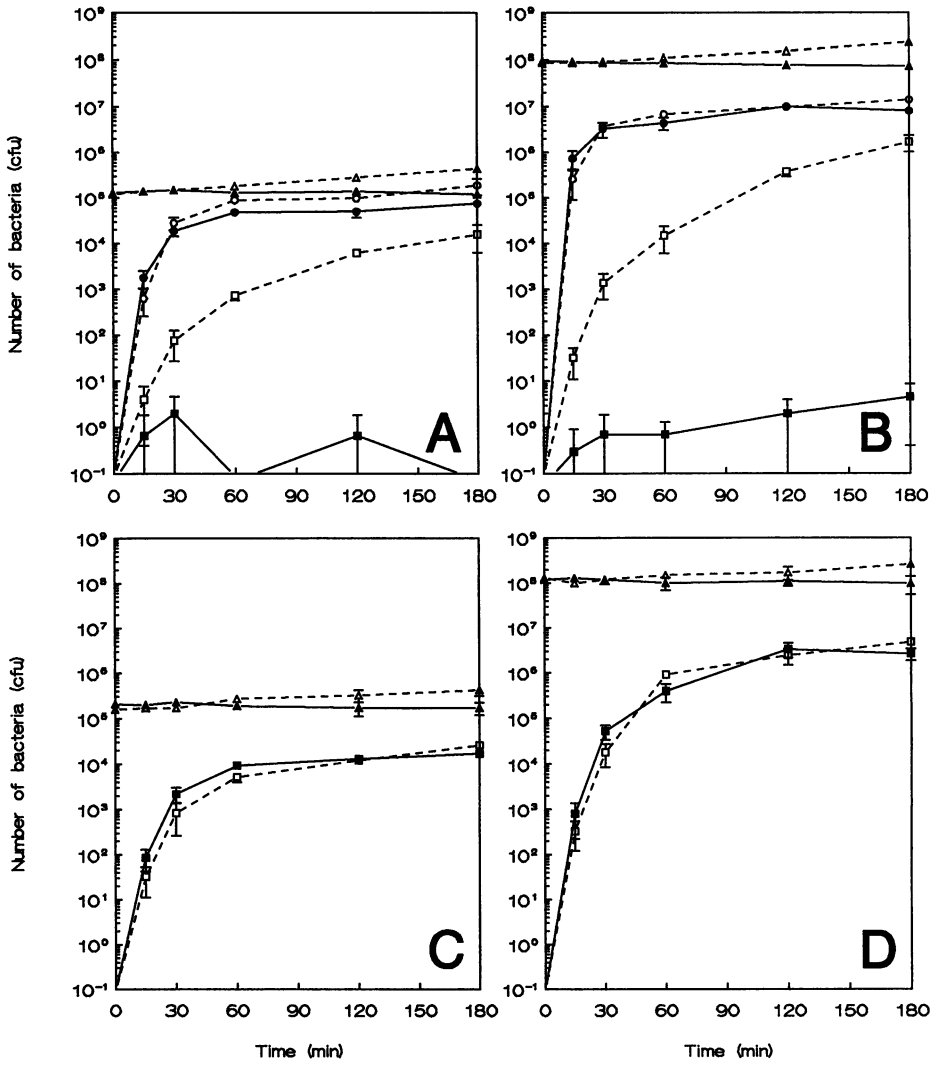

FIG. 2. Effects of the addition of chloramphenicol on the adhesion to and invasion of Int-407 cells by *S. typhimurium* C52. Symbols: ▲ and △, total number of bacteria; ● and ○, adhesive bacteria; ■ and □, colistin-resistant (intracellular or invasive) bacteria; △, ○, and □, no chloramphenicol added; ▲, ●, and ■, 40 μg of chloramphenicol per ml added at a *t* of −30 min. The data represent the statistical means of three experiments. Error bars (representing the sample standard error) were omitted if the bars were smaller than the symbols. (A and B) Int-407 cells infected with a logarithmic-phase culture of *S. typhimurium* C52 at MOIs of 0.1 and 100, respectively; (C and D) cells infected with a logarithmic-phase culture of *Y. enterocolitica* at MOIs of 0.1 and 100, respectively.

sion of mixtures of live and dead (glutaraldehyde-killed) bacteria. When a mixture of equal numbers of dead and live bacteria was added to the cells, the numbers of live bacteria (CFU) that adhered to the cells at a *t* of 180 min was approximately half that when live bacteria alone were added (data not shown). If 10^8 dead bacteria were added at a *t* of −30 min, the numbers of adherent live bacteria (CFU) were reduced 100- to 1,000-fold (data not shown).

To test whether de novo protein synthesis is needed in the adhesion or in the penetration stage of the invasion, we performed adhesion assays in the presence of chloramphenicol. The addition of chloramphenicol did not significantly alter the adhesion of the bacterium (Fig. 2A and B).

To separate adhesion from invasion, the adhesion experiments described above were performed with glutaraldehyde-fixated Int-407 cells. The finding that penetration but not adhesion is inhibited by chloramphenicol enabled us to measure adhesion to live Int-407 cell. Confluent monolayers of Int-407 cells were infected with bacteria (MOI = 0.1 to 1,000) to which chloramphenicol was added at a *t* of −30 min. The results of these experiments (Fig. 4) show that there is no significant difference in the adhesion rate or the maximum number of adhesive bacteria between bacteria adhering to fixated or live Int-407 cells.

Adhesion and invasion studies with other *S. typhimurium* strains. To exclude that strain C52 is a variant with exceptional invasive properties, we tested the invasion and adhesion of the frequently used SR11 and SL1344 strains. The obtained results (Fig. 5) show that all three strains behave similarly. To validate the use of colistin for killing the extracellular gentamicin-resistant C52, we assayed the invasion of SR11 and SL1344 with either colistin or gentamicin as the selective antibiotic. The results (Fig. 5) show that both antibiotics are equally effective. As an additional control, we assayed the three strains described above and an additional 27 independent field isolates of *S. typhimurium* (MOI = 10)

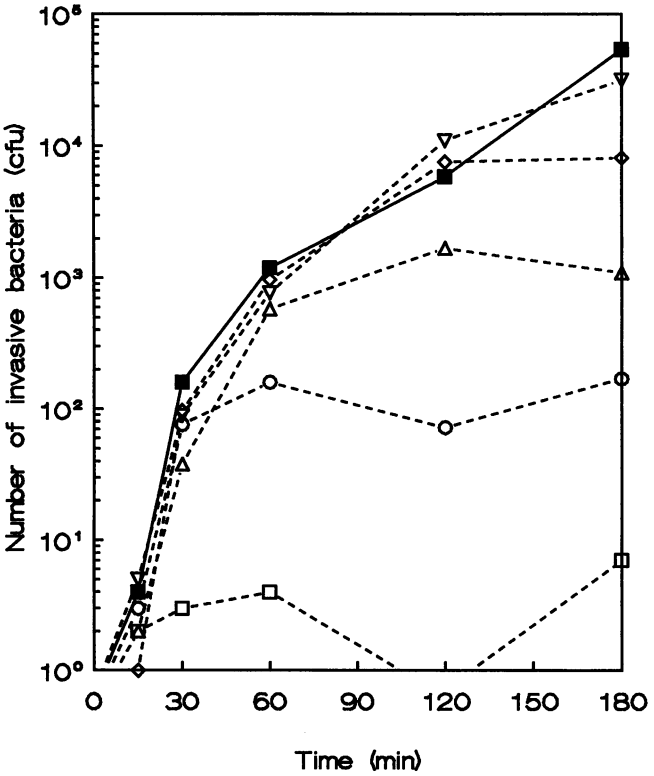

FIG. 3. Effect of the time of addition of chloramphenicol on the invasion of Int-107 cells by *S. typhimurium* C52. Chloramphenicol (40 μg/ml) was added to the inoculum at different time points. The inoculum (10^6; MOI = 1) consisted of a logarithmic-phase culture of strain C52. Symbols: closed squares, control with no chloramphenicol added; open symbols, chloramphenicol added at *t* of 15 (□), 30 (○), 60 (△), 120 (◇), and 180 (▽) min. Addition of chloramphenicol at *t* of 30, −15, 0, and 5 min did not result in colistin-resistant (intracellular or invasive) bacteria in this experiment. These results are not represented in this graph. The data are from one of two independent experiments.

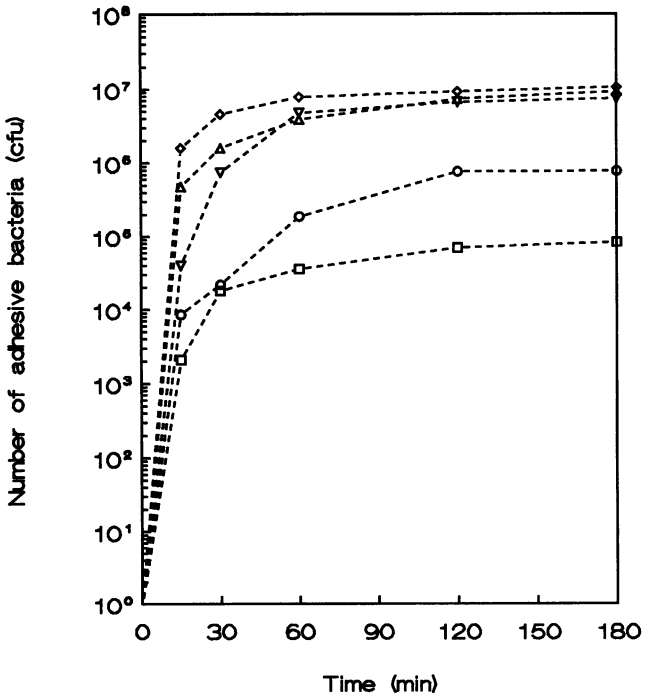

FIG. 4. Adhesion of *S. typhimurium* C52 to live Int-407 cells. Chloramphenicol (40 μg/ml) was added to the inoculum (consisting of a logarithmic-phase culture of strain C52) at a *t* of 30 min. This prevents the bacteria from entering the cells (Fig. 3). The symbols represent the number of cell-associated bacteria (CFU) found with cells infected at an MOI of 0.1 (□), 1 (○), 10 (▽), 100 (△), or 1,000 (◇). The data are from one of two independent experiments.

to Int-407, HeLa, and HEp-2 cells in a simplified assay where adhesion was measured at a *t* of 30 min and invasion was measured at a *t* of 60 min. The levels of adhesion and invasion of these strains and the effect of chloramphenicol addition were similar to those of *S. typhimurium* C52 (data not shown).

DISCUSSION

Our results can be summarized as follows. (i) The fraction of the inoculum that adheres or invades is related to the MOI. (ii) When added at equal MOIs, there are no significant differences in adhesion and invasion between logarithmic- and stationary-phase bacteria. (iii) Adhesion occurs instantly. (iv) There is a maximum number of bacteria that can bind to a cell, probably because the surface of Int-407 cell contains only a limited number of receptors for the adhesion of *S. typhimurium*. (v) Adhesion occurs even when both bacteria and cells are dead, but invasion requires metabolic activity from bacteria and cells. Below, we will discuss these conclusions separately and also address the differences between our results and the published data of others.

Most studies on *S. typhimurium* adhesion and invasion (5, 7, 9, 12, 15, 18) express the adhesion and invasion rates as the fractions of the total number of bacteria added, often without reference to the MOI. We showed that the MOI has a clear effect on the fraction of the inoculum adhering to (or invading) the cell (Fig. 1). This makes it difficult to compare data. Where possible (5, 9, 28), we calculated from these published data the number of bacteria that were bound or had invaded per cell. A comparison of the data calculated from Schiemann and Shope and Francis et al. (9, 28) with ours reveals that we have a 10- to 20-fold higher invasion rate. Several studies on the invasion of *S. typhimurium* use a low-speed centrifugation step to promote the invasion. Even compared with these studies (5), our invasion rates are two- to fivefold higher. Since we define invasion as a two-stage process where adhesion precedes penetration and centrifugation obscures any active contribution of the bacterium in the first step, we did not extensively test the effects of centrifugation. The use of centrifugation to promote invasion not only increased our invasion rates 4- to 20-fold (*t* = 180 min; MOI = 100 and 0.1) but also accelerated the process, resulting in 10 to 90% (MOI = 100 and 0.1) of the inoculum being internalized at *t* = 30 min (data not shown). Comparing these data with published data again shows that we obtained high invasion rates. Differences in observed invasion rates may be caused by differences in the media or in the conditions used to grow the cells and bacteria.

We found no significant differences in adhesion and invasion between logarithmic- and stationary-phase bacteria. Lee and Falkow (18) and Ernst et al. (5) reported a clear effect of the growth phase on invasion. In their experiments (5, 18), differences in the MOI and bacterial growth during the experiment were not fully accounted for, but they might

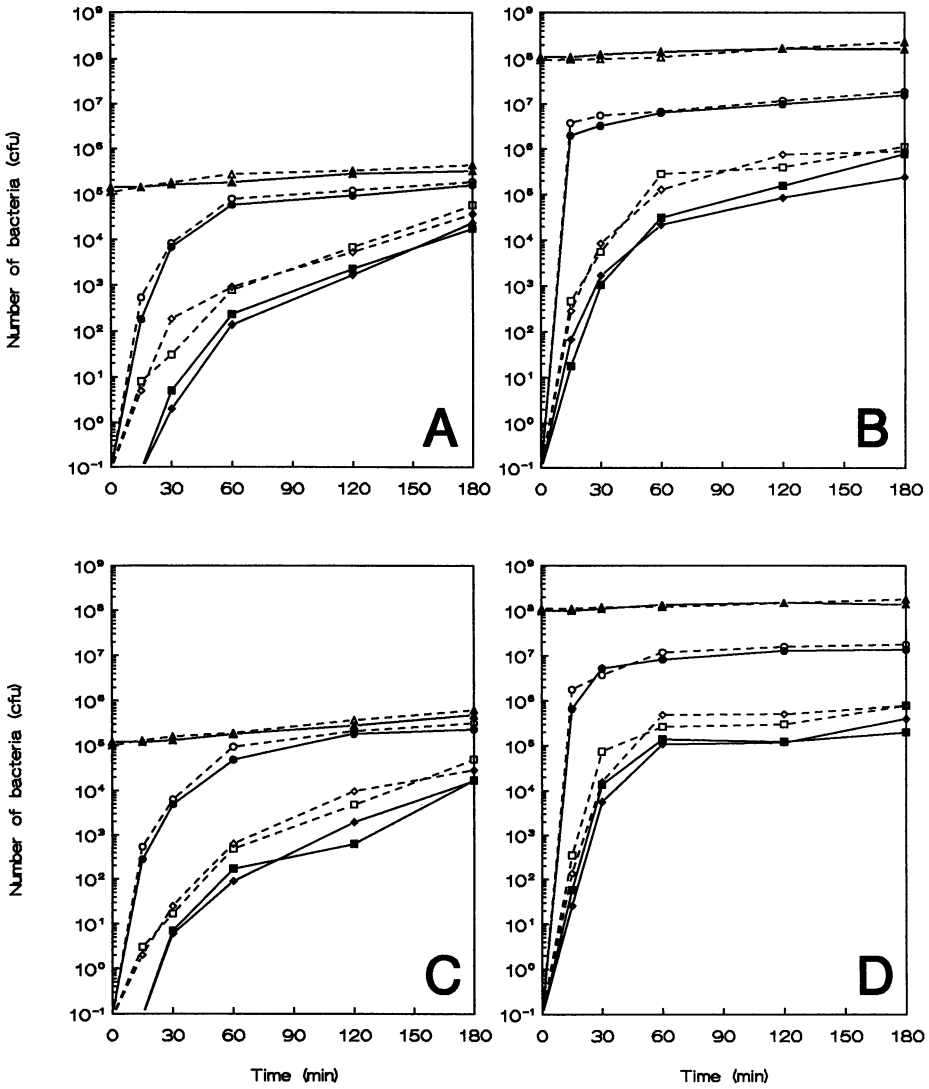

FIG. 5. Adhesion to and invasion of Int-407 cells by *S. typhimurium* SL1433 and SR11. Symbols: ▲ and △, total number of bacteria; ● and ○, adhesive bacteria; ■ and □, colistin-resistant (intracellular or invasive) bacteria; ◆ and ◇, gentamicin-resistant (intracellular or invasive) bacteria; △, ○, □, and ◇, bacteria in mid-log phase; ▲, ●, ■, and ◆, bacteria in stationary phase. (A and B) Int-407 cells infected with a logarithmic-phase culture of *S. typhimurium* SR11 at MOIs of 0.1 and 100, respectively; (C and D) cells infected with a logarithmic-phase culture of strain SL1344 at MOIs of 0.1 and 100, respectively. The data are from one of two independent experiments.

well be responsible for the observed difference in invasion rate. Again, as mentioned above, various methods ranging from vigorous shaking (as we did) to standing overnight cultures are used to obtain stationary-phase cultures. Besides altering the CFU per milliliter of the inoculum, these methods will also influence other parameters like the oxygen content and pH of the medium. Although we did not systematically address this, the reported (5, 18, 28) increased invasion rate of anaerobically versus aerobically grown bacteria might partially (or completely) be attributed to differences in MOI. Schiemann and Shope (28) reported a fivefold increase in invasiveness between anaerobic and aerobic bacteria. From their data, we calculated that they used MOIs of 0.2 and 1 for anaerobic and aerobic bacteria, respectively. If we express our invasion rate as a percentage of the inoculum (as they did), this difference in MOI alone would result in a fivefold decrease of the invasion rate.

Finlay et al. (7) observed a 2-h lag time before adhesion occurred. While this was originally interpreted as an indication for the requirement of de novo protein synthesis, it is now attributed to the use of bacteria in a state equivalent to a stationary-phase culture (9). As discussed above, we did not observe significant differences in adhesion or invasion rates between bacteria derived from either stationary- or logarithmic-phase cultures. We found that, even with bacteria from a stationary-phase culture at a low MOI, maximum numbers of adhesive bacteria were found within 30 min (Fig. 1A). This indicates that the factor(s) necessary for bacterial adhesion is already present at a *t* of 0 min, on both the bacteria and the cells.

Our data show that there is a maximum of approximately 10 bound bacteria per Int-407 cell. This maximum occurred independently of the bacterial strain or cell line used. To our knowledge, there are no previous reports on the occurrence of such a maximum. At an MOI of 0.1, this maximum corresponds to 200% of the bacteria in the initial inoculum

(50% if we correct for bacterial growth), while at an MOI of 100, less than 1% adhere. This indicates that the maximum number of bacteria that can bind per cell is not determined by the bacterium but by the cell. Scanning electron micrographs and light microscopic examination (data not shown) of cells binding the maximum number of bacteria showed that only a small part of the cell surface is covered by the adherent bacteria. This indicates that the cellular receptor(s) is present in limited numbers or it is located exclusively at specific areas of the cell surface.

To separate adhesion from invasion, we performed our adhesion experiments with glutaraldehyde-fixated Int-407 cells. Comparison of the adhesion of *S. typhimurium* to fixated Int-407 cells with adhesion to live Int-407 cells shows that adhesion to fixated Int-407 cells reflects the interaction with live cells. In contrast to adhesion where a maximum number of adhesive bacteria was observed, no maximum number was observed for invasive bacteria. Apparently, bacteria that are bound to the cell are not immediately internalized. This may be caused by a limiting supply of bacterial and/or cellular factors involved in penetration. We have shown that invasion of Int-407 cells by *S. typhimurium* C52 has an absolute requirement for ongoing protein synthesis. Since the same results were obtained with the protein synthesis inhibitor spectinomycin and the RNA synthesis inhibitor rifampin (not shown), it is unlikely that it is an artifact induced by the antibiotic. In addition, the control with *Y. enterocolitica* (Fig. 2), where invasion is not influenced by the addition of chloramphenicol, also excludes that some unknown effect of chloramphenicol on Int-407 cells is responsible for the inhibition of invasion by *S. typhimurium*. The obtained data on chloramphenicol, spectinomycin, and rifampin blocking invasion but not adhesion provide additional evidence for the two-stage model of invasion. Additional evidence comes from the finding that dead (glutaraldehyde-killed) bacteria not only compete with adhesion of live bacteria but also interfere with the invasion of live bacteria (data not shown). The addition of chloramphenicol at various stages during the invasion process (Fig. 3) shows that addition up to 15 min after the bacteria were added to the cells completely prevented the bacteria from entering the cells. This indicates that the first 15 min of the initial contact between bacteria and cells is needed to initiate the synthesis of the proteins that are required for invasion. Since prolonged (24-h) incubation with chloramphenicol does not affect the viability of intracellular bacteria (data not shown), the de novo-synthesized invasion-related proteins are required for penetration and not for intracellular survival. When added after a t of 15 min, chloramphenicol immediately stopped further invasion. There are several explanations for this observation. (i) Invasion requires de novo synthesis of invasion-related proteins, and chloramphenicol inhibits this synthesis. (ii) The proteins needed for invasion are very unstable. (iii) These proteins are produced in limiting amounts. (iv) The cells are not always capable of internalizing bacteria, and thus they delay the penetration. (v) The production of the proteins required for the invasion may occur only during specific stages of the bacterial life cycle, or bacteria may need a special signal from the cell before they start the synthesis of these proteins. The observed time course of the invasion kinetics suggests that the last possibility may be true.

The observed immediate effect of protein and RNA synthesis inhibitors on the invasion is not completely in agreement with the data presented in the article of MacBeth and Lee (22) that appeared during the revision of this manuscript. Although they also report a clear effect of inhibition of protein synthesis on the invasion rate of *S. typhimurium*, they observed only a gradual decrease in invasiveness that became more pronounced as the preincubation time with the antibiotic was prolonged. Apparently, in their assay, the half-life of the essential invasion factor(s) is distinct from ours, either because different factors are limiting or, in their system, these factors are more stable.

We have recently cloned *S. typhimurium* genes that seem to encode adhesion- and invasion-induced proteins that are potentially involved in the penetration of eukaryotic cells and are currently characterizing these clones.

ACKNOWLEDGMENTS

We thank J. Meijerink, M. S. Evers, and L. E. G. M. de Groot for technical assistance.

J.G.K. was supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences and by Solvay/Duphar B.V., Weesp, The Netherlands.

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