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NOTES

Prolonged Inhibition of Bacterial Protein Synthesis Abolishes *Salmonella* Invasion

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We have found that prolonged inhibition of bacterial protein synthesis abolishes the ability of *Salmonella typhimurium* to enter HEp-2 cells. Our results suggest that an essential invasion factor has a functional half-life that is seen as a gradual loss of invasiveness in the absence of protein synthesis. Therefore, *Salmonella* invasiveness appears to be a transient phenotype that is lost unless protein synthesis is maintained. This finding may explain why salmonellae grown to stationary phase lose their ability to enter cultured cells. In addition, a short-lived capacity to enter cells may be important during infection so that bacterial invasiveness is limited to certain times and host sites during pathogenesis.

The ability of salmonellae to enter cultured epithelial cells is modulated by the bacterial growth state, such that bacteria growing under low-oxygen and high-osmolarity conditions are induced for invasion (2, 9, 18, 21). Contact between such induced bacteria and epithelial cells results in changes within the epithelial cell, including disruption of the cellular brush border, protein phosphorylation, calcium ion influx, and polymerization of actin (5, 7, 10, 11). As a consequence of these cellular events, the salmonellae are internalized by the epithelial cell in a membrane-bound vacuole. Apparently, extracellular interaction with salmonellae results in the transduction of a signal into the epithelial cell which initiates intracellular changes and allows bacterial invasion.

Experimental evidence suggests that the induced, invasive salmonellae possess all of the bacterial factors required for the invasion process. For example, the entry process occurs rapidly, within minutes (8). In addition, once salmonellae are grown under inducing conditions, bacterial protein synthesis is not required for invasion (18). However, we and others have found that salmonellae which have been killed by incubation with gentamicin or by incubation at 55°C, even when initially grown under the inducing conditions, are not able to enter cultured epithelial cells (reference 7a and our unpublished observations). These results seem to contradict the conclusion that induced bacteria are inherently able to enter epithelial cells. We decided to investigate why bacterial invasiveness is lost when bacteria are killed with gentamicin or heat.

The standard protocols for killing bacteria with gentamicin or heat require treatment for 2 h or 1 h, respectively (13, 17). In order to control for the lack of bacterial growth during the treatment periods, we incubated bacteria in parallel samples with inhibitors of protein synthesis. To our surprise, we found that prolonged treatment of *Salmonella typhimurium* with inhibitors of protein synthesis abolishes invasion, even though the bacteria are not killed.

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Initially, *S. typhimurium* SL1344 (12) was grown under inducing conditions; 5 ml of Luria-Bertani broth inoculated with ~10^4 CFU/ml was incubated overnight at 37°C without agitation (18, 19). Bacteria were then inoculated into tissue culture medium (Eagle minimum essential medium with Earle's salts and 5% fetal bovine serum; Whittaker Bioproducts, Walkersville, Md.) in the presence or absence of protein synthesis inhibitors at 37°C in 5% CO_2–95% air. At various times after incubation, we evaluated bacterial invasiveness by transferring each sample to wells containing HEp-2 monolayers (ATCC CCL 23) (18). Bacteria were allowed to enter the cells during a 30-min incubation in the presence or absence of inhibitor. Then, the infected monolayers were washed five times with phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. The monolayers were then treated with tissue culture medium containing 100 μg of gentamicin per ml for an additional 90 min. Since gentamicin cannot cross the epithelial cell membrane, extracellular bacteria were killed, and we quantitated intracellular bacteria by plating for viable CFU (22). In order to be able to compare the invasion assays, we also quantitated the total bacteria present at the end of the invasion period. Basically, the number of CFU was determined for parallel samples, except that these samples were not incubated with HEp-2 cells. In this way, bacterial invasion was calculated as the CFU of intracellular bacteria divided by the CFU of total bacteria in the assay. In order to have similar numbers of total bacteria in each assay (~3×10^7 CFU), we compensated for any bacterial growth in samples incubated for prolonged periods without protein synthesis inhibitors by decreasing the initial bacterial inoculum.

Our results show that addition of chloramphenicol (100 μg/ml) or tetracycline (50 μg/ml) does not immediately affect the ability of *S. typhimurium* to enter HEp-2 cells (Fig. 1). Bacteria are fully invasive even when assayed in the presence of inhibitors. However, prolonged preincubation with inhibitors (90 min) reduces bacterial invasiveness 100- to 1,000-fold compared with that of the untreated control bacteria or compared with that of bacteria treated with inhibitors for short periods. To ensure that our assay is truly

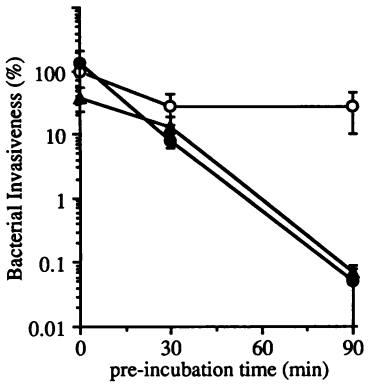

FIG. 1. Effect of preincubation with protein synthesis inhibitors on *S. typhimurium* invasion. Bacteria were preincubated and assayed in tissue culture medium alone (open circles), in tissue culture medium containing 100 μg of chloramphenicol per ml (closed circles), or in tissue culture medium containing 50 μg of tetracycline per ml (triangles). Bacterial invasiveness was measured as the ability of SL1344 to enter HEp-2 cells during a 30-min incubation. Relative values are plotted so that the invasiveness of bacteria assayed in tissue culture medium alone without preincubation equals 100%. Error bars represent the standard deviation of data from at least three separate assays. In six different experiments, the actual percentage of untreated SL1344 inoculum that entered HEp-2 cells in 30 min was 0.28, with a standard deviation of 0.11.

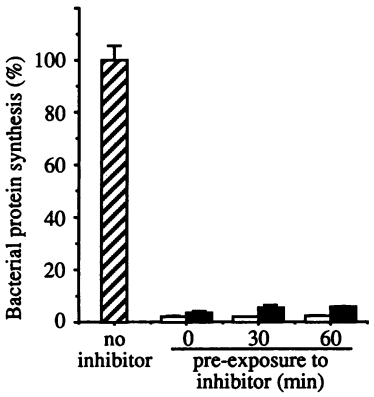

FIG. 2. Effect of preincubation with protein synthesis inhibitors on *S. typhimurium* protein synthesis. Bacteria were assayed for the ability to incorporate [^{35}S]methionine into trichloroacetic acid-precipitable counts during a 30-min period. Bacteria were assayed in tissue culture medium alone (striped bar) or were preincubated and assayed in tissue culture medium containing 100 μg of chloramphenicol per ml (open bars) or 50 μg of tetracycline per ml (closed bars). Values are plotted so that the synthetic capacity of bacteria in tissue culture medium alone equals 100%.

representative of bacterial entry into HEp-2 cells, bacterial association and invasion were examined microscopically. Immediately after the 30-min infection period, HEp-2 monolayers were washed with PBS and stained to visualize bacteria. Examination of Giemsa-stained monolayers, as well as immunofluorescent intracellular versus extracellular bacteria (1), revealed that prolonged treatment with bacterial protein synthesis inhibitors does indeed abolish the ability of *S. typhimurium* to enter HEp-2 cells (data not shown).

Chloramphenicol and tetracycline inhibit the rate of bacterial protein synthesis. In order to examine the effects of these antibiotics on bacterial protein synthesis under our invasion assay conditions, we quantitated the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable counts. Basically, we incubated SL1344 in tissue culture medium containing protein synthesis inhibitors. At various times after incubation, we evaluated the ability of the bacteria to synthesize protein by addition of 10 μCi of [^{35}S]methionine for 30 min. Acid-precipitable protein was recovered on Whatman GF/F filters. We found that the ability of chloramphenicol (100 μg/ml) and tetracycline (50 μg/ml) to inhibit bacterial protein synthesis does not require preincubation (Fig. 2). Therefore, bacterial protein synthesis is not required for invasion. Furthermore, the time-dependent loss of invasion (Fig. 1) is not due to a delay in inhibition of protein synthesis.

Although bacterial invasion factors are required for the invasive phenotype, other bacterial properties, such as chemotaxis and motility, influence the ability of salmonellae to enter cultured epithelial cells (3, 14–16, 20). Thus, in order to examine whether protein synthesis inhibitors reduce invasion by affecting bacterial flagella or motility, we conducted an experiment with an isogenic *S. typhimurium* strain, SL7111 (B. A. D. Stocker, Stanford University School of Medicine) (4), that lacks flagella and consequently is nonmotile. Bacteria were grown and treated with chloramphenicol as described above; however, in order to evaluate their invasiveness, bacteria were centrifuged onto the HEp-2 monolayer. As determined by gentamicin survival, as well as microscopic examination of infected monolayers, prolonged incubation with chloramphenicol abolished the ability of SL7111 to enter HEp-2 cells (data not shown). Thus, the effect of chloramphenicol on invasion is independent of bacterial flagella and motility.

Our results show that prolonged inhibition of bacterial protein synthesis abolishes the ability of *S. typhimurium* to enter cultured epithelial cells. Furthermore, de novo bacterial protein synthesis is not required for invasion. Our findings suggest that there is a steady loss of an essential bacterial invasion factor in the absence of protein synthesis. Possibly, an invasion factor is secreted from an intracellular pool and, in the absence of continual protein synthesis, the pool is depleted. Or, alternatively, an essential invasion factor may be labile and its half-life is reflected as the gradual loss of invasiveness in the absence of protein synthesis. Regardless, these results explain why bacteria killed with gentamicin lose their ability to enter cultured cells; prolonged treatment with gentamicin inhibits protein synthesis and kills the bacteria as well. Heat treatment also inhibits protein synthesis, and high temperatures may even increase loss of a labile invasion factor. Further experiments are required to determine whether bacteria killed without prolonged treatments are invasive or not.

Although bacteria in stationary phase are physiologically distinct from bacteria treated with protein synthesis inhibitors, the balance of synthesis and loss of an invasion factor might be affected similarly by cessation of growth in stationary phase and by treatment with protein synthesis inhibitors. Thus, our hypothesis may also account for why salmonellae grown to stationary phase lose their invasiveness (2, 18).

Interestingly, bacterial protein synthesis has been shown to be required for maintenance of *Salmonella choleraesuis* adherence to epithelial cells. Basically, treatment with chloramphenicol for 1 h reduced bacterial adherence to fixed cells four- to fivefold (6). We did not examine the effect of inhibition of protein synthesis on *S. typhimurium* adherence to fixed HEp-2 cells. However, recent studies show that the

loss of bacterial invasiveness during stationary phase cannot be restored by increasing *S. typhimurium* adherence to cells (8). Therefore, one bacterial factor lost during stationary phase, and possibly during inhibition of protein synthesis, appears to be essential for invasion at a step after bacterial contact with cells.

Our results lead to interesting speculation about what occurs during pathogenesis. We have proposed that invasion factor expression might be induced in the intestinal lumen to allow salmonellae to enter intestinal epithelial cells (18). However, once the bacteria are within cells or are across the epithelial barrier, the capacity to enter cells may no longer be a desirable property. Thus, it is possible that after salmonellae leave the intestinal lumen, they stop expressing invasion factors and, as we have seen after inhibition of protein synthesis in vitro, the bacteria rapidly lose their invasive phenotype in vivo. In this way, regulated expression of invasion factors, together with the short half-life of invasion factor function, may be an important mechanism to limit *Salmonella* invasion to certain times and host sites during pathogenesis.

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