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Invasion and Replication of *Salmonella typhimurium* in Animal Cells

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A total of 81 avirulent Tn*10* insertion mutants of *Salmonella typhimurium* have previously been described. These mutants were selected for the inability to survive in murine macrophages. We have characterized the abilities of the most avirulent of these mutants to adhere to, invade, and replicate in both macrophages and nonphagocytic epithelial cells. The results suggest that most mutants contain a defect that is specific to survival within professional phagocytes. These mutants invaded and replicated normally within nonphagocytic human colon adenocarcinoma cells (Caco-2) but did not survive in the macrophage cell line J774. One mutant invaded both macrophages and epithelial cells much less efficiently than the parental strain. The defect associated with this mutant appears to be a result of decreased adherence to animal cells.

Salmonella typhimurium is a gram-negative, facultative intracellular bacterium which can cause a lethal infection in susceptible strains of mice. The systemic infection induced in mice by *S. typhimurium* has many of the hallmarks of the human disease induced by *S. typhi*, including anorexia, dissemination through the reticuloendothelial system, splenomegaly, and diarrhea (14). We have chosen to study how *Salmonella typhimurium* survives within the macrophage since the spleen and splenic macrophages are primary sites of *S. typhimurium* replication in infected mice. We reported previously the isolation of mutants of *S. typhimurium* that were unable to survive within murine macrophages (4). Experiments presented here further characterize 23 of the least virulent mutants (50% lethal doses [LD_{50}s] at least 4 orders of magnitude greater than that for the virulent parent) for the ability to invade and replicate within nonphagocytic cells.

MATERIALS AND METHODS

LD_{50}. The number of bacteria which killed 50% of the injected mice was determined as described by Reed and Muench (16). Groups of five mice were injected intraperitoneally with 0.2 ml of bacteria suspended in physiologic saline. The number of bacteria injected was confirmed by plating a portion of the diluted bacteria on Luria-Bertani (LB) agar.

Cell culture. All tissue culture reagents were obtained from GIBCO Laboratories, Grand Island, N.Y., or from the tissue culture facility at Scripps Clinic. J774 cells were grown in Dulbecco minimal essential medium supplemented with 5% fetal calf serum at 37°C with 5% CO_2 (5, 7). For the invasion and replication assays, the adherent cells were removed by being treated with phosphate-buffered saline (PBS) containing 0.5 mM EDTA and reseeded either onto ethanol-washed microscope cover slips (round 14-mm-diameter no. 1; Bellco Glass, Inc., Vineland, N.J.) in a 24-well microdilution dish or directly into a microdilution dish. Caco-2 cells were grown in the same medium and transferred following trypsinization.

Opsonization of bacteria. The anti-*Salmonella* antibody used for the opsonization study was produced in mice. Mice which survived LD_{50} analysis with a mutant strain were challenged with 10^6 parental bacteria (14028r). Sera from mice surviving this challenge were pooled. This antiserum was titered against sonicated 14028r cells in an enzyme-linked immunosorbent assay, and a titer of 1:20,000 was obtained. This antiserum was equally reactive with MS3792r, as determined by enzyme-linked immunosorbent assay. For opsonization studies, bacteria were incubated with this mouse anti-*Salmonella* antiserum for 30 min on ice. Bacteria were then washed with saline and used in a macrophage invasion assay.

Invasion and intracellular replication assays. Table 1 lists the mutants employed in this study and their LD_{50}s. The original assay for bacterial invasion was modified by briefly centrifuging the bacteria onto the eucaryotic cell monolayer. This step facilitated contact between bacteria and macrophages, giving more-reproducible results than did merely incubating the bacteria and cells together. Events took place more synchronously, thus allowing us to distinguish between uptake and intracellular replication by comparing the results at various times after infection.

Fresh overnight bacterial cultures were centrifuged onto a monolayer of J774 (a murine macrophage cell line) or Caco-2 cells (a human adenocarcinoma cell line). The usual ratio of added bacteria to tissue culture cell was about 100:1. The microdilution dish containing the monolayer and bacteria was incubated at 37°C with 5% CO_2 for 30 min (J774) or 2 h (Caco-2). Nonadherent or nonphagocytosed bacteria were then removed by being washed with PBS several times. The cells were lysed immediately with detergent as a crude measure of adherence or incubated for various times to allow cell invasion and intracellular replication to take place. If incubation continued, gentamicin was added at 100 μg/ml for 30 min to kill extracellular bacteria. The cells were then washed, and fresh medium supplemented with 2 μg of gentamicin per ml was added to prevent extracellular replication. The macrophages were lysed with detergent, and the number of intracellular bacteria was determined by plating on LB agar plates and counting CFU.

We have used gentamicin to inhibit extracellular replication. Several others have already reported that this antibiotic does not affect growth of intracellular bacteria (13, 17). To verify this finding, in our own system we have compared replication of the parent strain, 14028r, in J774 cells with and

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TABLE 1. LD_{50}s of *S. typhimurium* mutants used in this study

Mutant strain	LD_{50}[a]
MS14028r	$<1 \times 10^3$
MS1	3×10^6
MS161	$>1 \times 10^7$
MS290	$\sim 1 \times 10^7$
MS4602	4×10^6
MS1321	$\sim 1 \times 10^6$
MS1405	$\sim 1 \times 10^6$
MS1446	5×10^6
MS1521	9×10^6
MS1523	5×10^6
MS1592	1×10^6
MS1633	1×10^6
MS1668	1×10^6
MS3575	$>1 \times 10^6$
MS3747	$\sim 1 \times 10^6$
MS3792	$>1 \times 10^6$ (r) $\sim 1 \times 10^5$ (s)
MS4179	$>1 \times 10^6$
MS4252	1×10^7
MS6290	2×10^6
MS7481	$\sim 1 \times 10^7$
MS7953	$>1 \times 10^7$
MS8194	$\sim 5 \times 10^6$
MS8282	$>1 \times 10^6$
MS8467	4×10^6
MS8537	$>1 \times 10^6$

[a] As determined by using the *Salmonella* sp.-sensitive mouse strain BALB/c. The LD_{50}s for the parental and mutant strains were determined by the method of Reed and Muench (16) but are only approximate because of the low number of mice (6 to 15) that could be used for these studies. Greater than or less than symbols mean that all mice either survived or died from the dose indicated. All results are for the rough derivatives of the strains as they were initially reported by Fields et al. (4). The LD_{50} for the smooth derivative of MS3792 is reported as well as that for the rough.

without 100 μg of added gentamicin per ml. Without gentamicin, it was necessary to wash the cells several times during a 6-h incubation to avoid large numbers of extracellular bacteria. We obtained identical growth curves with or without added gentamicin (data not shown).

Measurement of attachment and cell invasion in macrophages by immunofluorescence microscopy. Immunofluorescence was used to distinguish between attached and internalized bacteria. Approximately 5×10^5 J774 macrophages were seeded onto glass cover slips by the addition of a suspension of J774 cells in Dulbecco minimal essential medium to 6-well microdilution dishes containing the cover slips. Mutant and wild-type bacteria were grown overnight in LB medium, washed once in PBS, diluted in PBS, and spun onto the surface of the monolayer at a ratio of about 100 bacteria per macrophage by centrifugation at 1,200 rpm in an RT6000B desktop centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at room temperature. The J774 macrophages were washed three times to remove bacteria that were not attached to cells, and the plate was incubated for various times at 37°C with 5% CO_2. The number of input bacteria was separately quantitated by plating on LB agar and determining the number of CFU. At various times the cover slips were removed and washed once with PBS, and rabbit anti-*Salmonella* antibody was added. The cover slips were incubated for 30 min at room temperature and washed three times with PBS, excess liquid was blotted, and the cells were permeabilized with 4°C acetone for 10 min on ice. Internal (and external) *Salmonella* sp. cells were stained with mouse anti-*Salmonella* antibody. Finally, the second antibodies were added, which consisted of fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse antibodies. The cover slips were again incubated for 30 min at room temperature, washed three times in PBS, inverted onto a drop of 80% buffered glycerol on a glass slide, and sealed with nail polish. *Salmonella* sp. cells were visualized with a Microphot FX (Nikon). Bacteria within cells were stained red, while those attached to the outsides of cells were stained green and red.

Bacterial P22 transduction. Bacterial markers were transduced as described by Davis et al. (2). The method of preparing smooth derivatives of the rough isolates is described by Buchmeier and Heffron (1), as modified from the method of Gemski and Stocker (8).

RESULTS

***S. typhimurium* invasion and replication in nonphagocytic cells.** *S. typhimurium* Tn*10* mutants were screened for invasion of human colon adenocarcinoma epithelial (Caco-2) cells, as described in Materials and Methods, and the results of these assays are shown in Fig. 1. The fact that the bacteria were indeed intracellular was demonstrated by immunofluorescence microscopy (see Fig. 5 for an example; also see reference 7). All but two of the mutants (MS290 and MS3792) invaded Caco-2 cells at levels similar to that of the parent strain (14028).

To determine the efficiency of intracellular replication within Caco-2 cells, incubation of the mutant strains was continued for an additional 7 h. All but one of the mutants (MS3792) in this study appeared to replicate at rates similar to or greater than that of the parent strain within Caco-2 cells (Fig. 2). Several repetitions of these experiments have demonstrated that the lower number of intracellular bacteria seen for MS3792 was reproducible but not the higher number observed for several of the other mutants. It is likely that the lower number of intracellular MS3792 bacteria observed even after 8-h incubation reflects the low rate at which this mutant initially invades cells, since the few bacteria which did enter after 2 h increased in number over time (Fig. 1 and 2).

Invasion and replication in the macrophage cell line J774. Microscopic analysis suggested that *S. typhimurium* was present within J774 macrophages within minutes after the addition of bacteria to macrophage cultures. Therefore, the initial incubation time was lowered to 30 min for studies with J774. Invasion assays demonstrated that all but two of the mutants invaded (or were phagocytosed by) J774 macrophages at the same rate as the parent strain (Fig. 1). The same two mutants, MS290 and MS3792, had decreased invasion levels in both J774 and Caco-2 cells, suggesting that the defect has a similar effect in both macrophages and epithelial cells.

In contrast to the invasion assays, the results for intracellular replication were very different for Caco-2 and J774 cells. All mutants in this study not only did not replicate within J774 but were killed (data not shown). These results were in agreement with results obtained by using thioglycolate-elicited macrophages (4).

Invasion frequencies in peritoneal macrophages. Our initial experiments showed that two mutants were taken up less efficiently by the macrophage cell line J774. To determine whether these mutants were also less efficient at invading fresh murine macrophages, we also tested thioglycolate- and Proteose Peptone-elicited peritoneal macrophages and bone marrow-derived macrophages for internalization with these

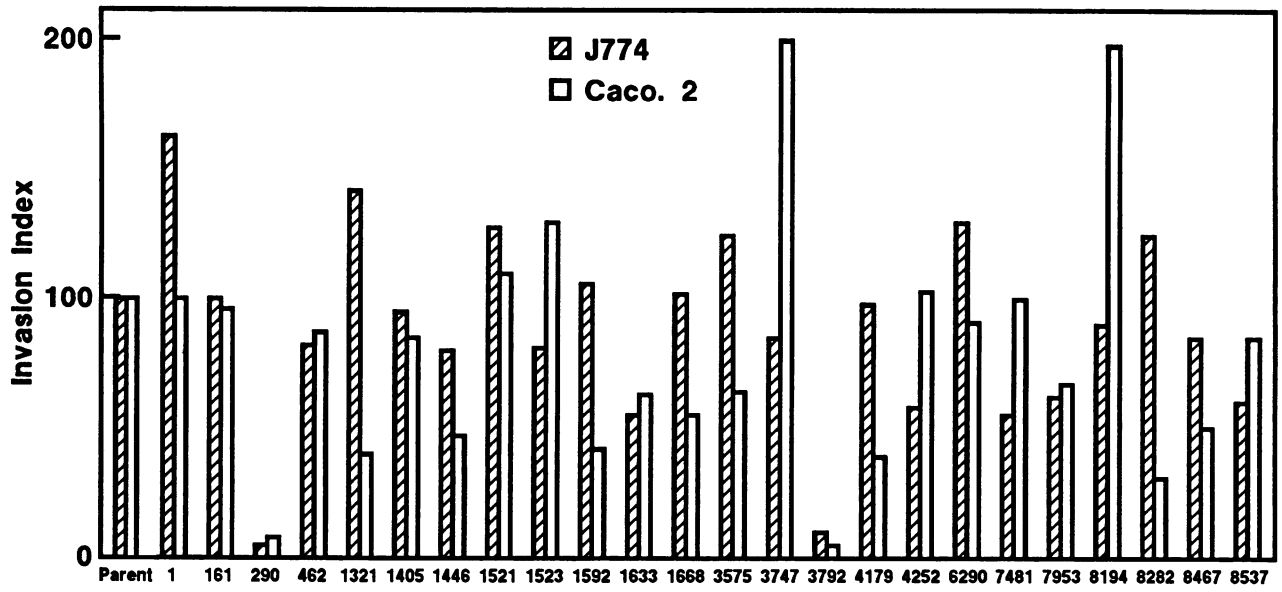

FIG. 1. *S. typhimurium* invasion of the macrophage cell line J774 and a human adenocarcinoma cell line, Caco-2. Invasion assays were carried out as described in Materials and Methods. Results are normalized to the number of the parent bacteria that invaded either Caco-2 or J774 cells. Results are internally consistent but not sufficiently reproducible from experiment to experiment to take the average. Results shown here are for a single experiment and show that all but two of the mutants invaded Caco-2 and J774 as well as the parental strain. The increases in invasion shown for a few of the mutants, such as MS3747, were not reproducible from experiment to experiment, although the decrease in invasion frequency for MS3792 was reproducible.

two mutants. The results obtained with elicited macrophages are shown in Fig. 3. Results with the bone marrow-derived macrophages were similar to those obtained with elicited macrophages (data not shown). The elicited macrophages were more microbicidal than J774, but MS3792 was not phagocytosed, nor did it invade any type of macrophage tested. Therefore, the defect in invasion is a constant property of the bacterium and not a function of the macrophage cell line or freshly isolated macrophages.

Phagocytosis of opsonized bacteria. Phagocytosis of bacteria by macrophages normally takes place via interaction between the bacteria and receptors on the macrophage surface. Opsonization increases the efficiency with which bacteria are taken up by macrophages since it provides additional ligands on the surface of the bacteria which can interact with receptors on the macrophage surface. If opsonization alters the mechanism by which *Salmonella* sp. enters the macrophage, opsonized mutants should enter

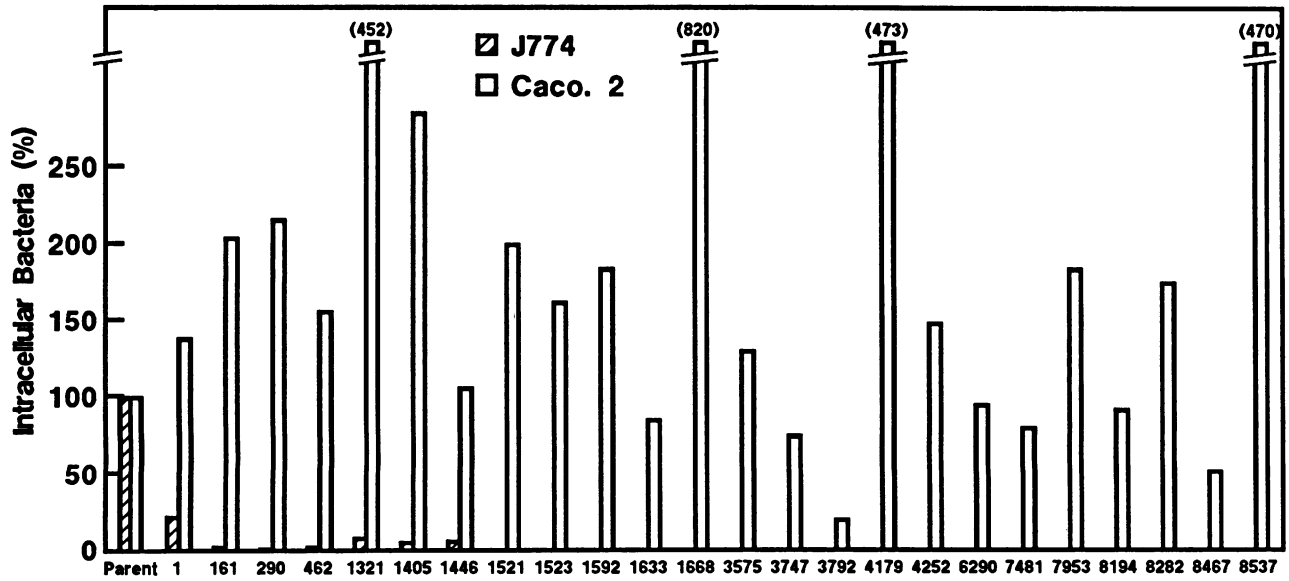

FIG. 2. *S. typhimurium* intracellular replication in the macrophage cell line J774 and in the human adenocarcinoma cell line Caco-2. Intracellular replication assays were carried out as described in Materials and Methods. Results are normalized to the number of parental bacteria that replicated in either Caco-2 or J774 cells. Results for intracellular replication in the macrophage cell line are not shown for all the mutants but were all low, less than 10% of that of the parental strain. Results are from a single experiment. No mutation showed a reproducible increase in replication over that of the parent.

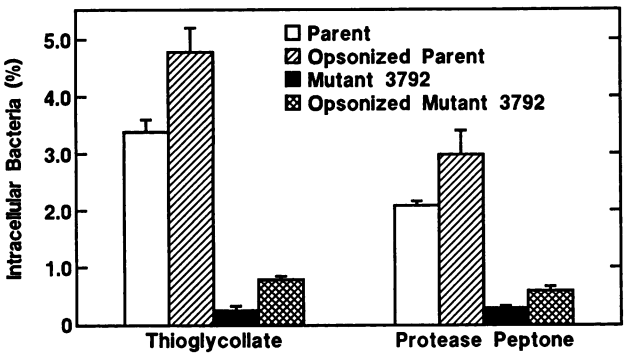

FIG. 3. Phagocytosis of mutant MS3792 in mouse peritoneal elicited macrophages. The macrophages were elicited and collected as described by Buchmeier and Heffron (1). Bacteria were opsonized by incubation with sera from mice previously infected with *Salmonella* sp. which contained high titers of anti-*Salmonella* antibody.

macrophages at rates similar to that of the parental strain. Opsonization of both the parental strain (14028) and MS3792 (rough), with the mouse anti-*Salmonella* antibody described in Materials and Methods, modestly increased the number of macrophage-associated bacteria (Fig. 3). However, opsonization of strain MS3792 did not compensate for the defect in invasion which this mutant possesses. The addition of sites of recognition (Fc regions of immunoglobulins) to MS3792 did not override the surface defects of MS3792 that interferes with invasion of elicited murine macrophages (Fig. 3).

Adherence of MS290 and MS3792 to J774 cells. Adherence of MS290 and MS3792 to J774 cells was measured by determining the number of cell-associated bacteria at various times after bacteria were centrifuged onto a monolayer of J774 cells. The number of cell-associated and internalized bacteria was quantitated by immunofluorescence. Bacteria and macrophages were incubated together for 5 to 10 min at 37°C with 5% CO_2. At this time the cells were washed three times with PBS to remove nonadherent bacteria. Extracellular bacteria were stained with a fluorescein-coupled second antibody. Intracellular bacteria were stained with a rhodamine-coupled second antibody. An association index was calculated as the total number of cell-associated bacteria divided by the number of input bacteria (Table 2). This index can be compared for the mutants and the parent bacteria. For example, at 5 min there were approximately 10% as many cell-associated MS290 bacteria as parent bacteria and 20% as many MS3792 bacteria (Fig. 4; Table 2). These results suggest that mutants MS290 and MS3792 possess defects that affect an early step in cell invasion, presumably the adherence step, although subsequent steps may be affected as well.

TABLE 2. Microscopic analysis of macrophage (Mϕ)-associated *S. typhimurium*

Time and strain	10^7 Input bacteria/cell	No. of Mϕ counted	No. of extracellular (green) bacteria	No. of intracellular (red) bacteria	Total no. of bacteria/Mϕ	Association index[a]
5 min						
14028r	7.95	205	1,483	364	9.01	100
290r	5.1	222	132	39	0.77	11
3792r	4.2	228	222	12	1.03	21
10 min						
14028r	7.95	208	690	108	3.84	100
290r	5.1	236	61	15	0.32	11
3792r	4.2	237	137	22	0.67	33

[a] Calculated as follows: (total number of bacteria per macrophage/number of input bacteria)/(number of parental bacteria per macrophage/number of input bacteria) × 100.

Effect of LPS on cell invasion frequencies. All of the 23 mutants in this study contain an intact core lipopolysaccharide (LPS) but are missing the O side chains. The parent of these mutants is thus rough but has a relatively low LD_{50} by intraperitoneal injection in mice (4). Finlay et al. (7) have reported that a complete LPS is necessary for *S. choleraesuis* to invade animal cells. To determine whether LPS could play a role in cell invasion for MS290 and MS3792, we transduced both of these mutations into cells in a smooth background and repeated the cell invasion assays (Table 3). The results show that the mutation in MS290 in the smooth background had only a marginal effect on cell invasion, while the mutation in MS3792 had a 10-fold effect in a smooth background and a 100-fold effect in a rough background.

DISCUSSION

This study confirms and extends earlier work on the isolation of macrophage-sensitive mutants of *S. typhimurium*. Of 23 mutants tested, 22 invaded and replicated normally within the human colon adenocarcinoma cell line Caco-2. We find that all but one of the mutants (MS3792 smooth) survive less well than the parent within the macrophage cell line J774, in contrast to within epithelial cells. This result suggests that most mutants contain defects that are specific to survival within professional phagocytic cells. The fact that the mutants behave similarly in J774 cells and thioglycolate-elicited peritoneal macrophages may suggest that J774 cells contain most of the same microbicidal products as do thioglycolate-elicited macrophages. This does not appear to be true for other types of macrophages (1).

One mutant, MS3792 (both the rough and smooth derivatives), reproducibly showed a much lower frequency of invasion into animal cells. This mutant also adhered to Caco-2 cells at a frequency which was less than 20% of the frequency of adherence by the parental strain. Finlay et al. (6) have shown that contact between *Salmonella* sp. and the eucaryotic cell induces expression of new proteins, some of which appear to be required for invasion of animal cells. They show that these new proteins fail to be induced in MS3792 and MS290. This complex phenotype (failure to both adhere and invade) might suggest that the strain is multiply mutant. This hypothesis can be ruled out because the strain contains only a single Tn*10* insertion, as determined by Southern hybridization (J. Lipps and F. Heffron, unpublished observations), and the defect can be transduced by P22 to other *Salmonella* strains by transducing tetracycline resistance.

Little is known about how *Salmonella* sp. invades animal cells and specifically what genes are required for cell invasion. By analogy with *Yersinia* spp., two or more separate cell invasion mechanisms are likely to be encoded. Intracellular replication within epithelial cells requires the products of several genes (9, 10). For example, laboratory strains of *Escherichia coli* containing the *Yersinia inv* gene invade animal cells but do not replicate in these cells, which suggests that additional genes besides the *inv* gene are required for intracellular replication (9, 10). What is known

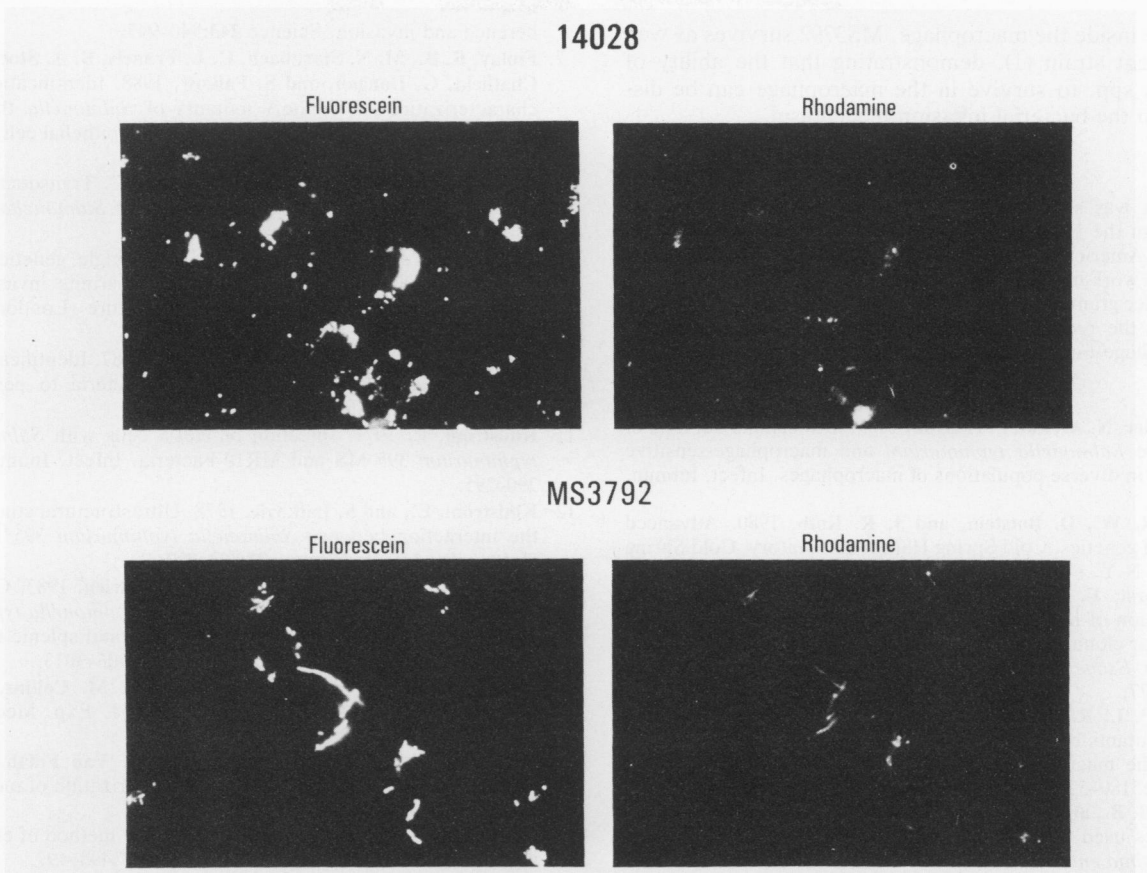

FIG. 4. Attachment of MS3792 to macrophages. Bacteria were allowed only a short time to adhere to J774 macrophages to avoid internalization. Double-label immunofluorescence microscopy was carried out as described in Materials and Methods. Extracellular cells were stained with fluorescein, and intracellular bacteria were stained with rhodamine. Little rhodamine staining of extracellular cells was obtained in this experiment, apparently because the primary antibody used to stain extracellular bacteria saturated most of the antibody sites. Macrophages were infected with approximately equal numbers of parental and MS3792 bacteria. Fewer MS3792 bacteria than wild type were observed attached to macrophages. The mutation in MS3792 appears to affect septation. Approximately 15% of the MS3792 bacteria were present in long filaments. We do not feel that this finding affects the conclusions of this study.

so far about *Salmonella* invasion and replication within eucaryotic cells is somewhat contradictory and probably reflects real differences between *Salmonella* serotypes and differences between target eucaryotic cells used in the studies. For example, *E. coli* which has received a single continuous piece of *S. typhi* DNA invades animal cells (3). The same continuous sequence is present in *S. typhimurium* but does not confer the cell invasion phenotype upon *E. coli*. In *S. cholerae-suis*, LPS is required for cell invasion because many mutants of *S. cholerae-suis* that do not invade MDCK cells are missing LPS (7). This finding is interpreted to mean that one component of the invasion machinery interacts with LPS or at least requires LPS for invasion of MDCK cells. Other investigators have found that rough strains of *S. typhimurium* invaded HeLa cells more efficiently than did smooth strains (11, 12). However, we find that rough derivatives of *S. typhimurium* invade animal cells as well as do smooth strains (Table 3). In view of this observation, the 10-fold difference in invasion frequency between smooth and rough derivatives of MS3792 may mean that there are two separate invasion mechanisms encoded by *S. typhimurium* and that MS3792 is deficient in only the LPS-independent one.

TABLE 3. Invasion of Caco-2 cells with rough and smooth derivatives of *S. typhimurium* ATCC 14028 and two MS mutants

Strain	% Invasion[a] ± SD
14028s	2.2 ± 0.9
14028r	3.4 ± 1.3
MS290s	0.91 ± 0.39
MS290r	1.0 ± 0.39
MS3792s	0.30 ± 0.10
MS3792r	0.033 ± 0.009

[a] Percent of input bacteria that have invaded Caco-2 cells after 1-h incubation, as described by Finlay and Falkow (5) and in Materials and Methods.

MS3792 is taken up poorly by macrophages even when opsonized. One normally thinks of phagocytosis as an active process on the part of the macrophage, initiated when the bacterium first binds to one of several macrophage receptors, including Fc, C3b, or the mannitol receptor (15). This binding then stimulates invagination and engulfment of the bacterium by the macrophage. How might a bacterial mutation affect phagocytosis? One explanation is that *S. typhimurium* encodes such an efficient cell invasion mechanism that most of the uptake we observe is not normal phagocytosis but, in fact, bacterium-mediated cell invasion. In this case, the residual invasion frequencies shown in Table 3 for MS3792 could simply reflect normal macrophage phagocy-

tosis. Once inside the macrophage, MS3792 survives as well as the parent strain (1), demonstrating that the ability of *Salmonella* spp. to survive in the macrophage can be dissected from the bacterial invasion mechanism.

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