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Invasion of Murine Intestinal M Cells by *Salmonella typhimurium inv* Mutants Severely Deficient for Invasion of Cultured Cells

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We have examined the role of the *Salmonella typhimurium inv* locus in invasion of the murine intestine. Previous studies have demonstrated that M cells within the lymphoid-follicle-associated epithelia are the primary site of intestinal invasion by *S. typhimurium*. In this study, we show that mutants possessing defects in one of two *inv* genes, *invA* or *invG*, which render them severely deficient for invasion of polarized epithelial MDCK cells, retain their ability to actively invade mouse Peyer's patch M cells. The interaction of these mutants with M cells was associated with apical membrane remodelling resembling that induced by wild-type strains. These data demonstrate that *Salmonella* invasion in vivo can proceed via mechanisms other than those previously defined in cultured cells.

Salmonellae are enteric pathogens which invade the intestinal epithelium to initiate disease. In mice, the primary sites of *Salmonella* invasion are the ileal Peyer's patches and possibly also the cecal lymphoid patches (1). The follicle-associated epithelium (FAE) overlying these gut-associated lymphoid tissues includes the specialized antigen-sampling M cells, which are a major site of invasion by diverse pathogens (15). Recent studies employing ligated murine intestinal loops suggest that M cells play a pivotal role in the pathogenesis of *Salmonella typhimurium*, since in the early stages of infection these cells are the primary site of bacterial invasion of the mouse intestine (3, 25, 28). M cells are not, however, the exclusive site of intestinal infection by *S. typhimurium*, as invasion of villous epithelia has been demonstrated in guinea pigs and rabbits (35, 36).

Interaction of salmonellae with intestinal M cells is accompanied by loss of cell surface microvilli and remodelling of the apical membrane to form structures resembling membrane ruffles (3, 25, 28). Similar changes accompany interaction of salmonellae with a variety of cultured cell lines and are believed to be critical for invasion (6–8). Although intracellular signalling is implicated in the triggering of membrane ruffling and invasion, the precise mechanisms underlying these events remain obscure, and it is possible that the mechanisms of invasion differ among cell types (9).

The genetic basis for *Salmonella* invasion is complex. Mutagenesis studies have demonstrated that attachment and entry are genetically separate events (11, 16), and a number of loci necessary for internalization of salmonellae by cultured epithelial cells have been identified (see, for example, references 10, 11, 18, 29, and 32). These loci include *inv* (11), which contains several genes whose mutation affects the ability of *S. typhimurium* to invade cultured epithelial cells without affecting bacterial adhesion. Products of the *inv* locus and neighboring regions of the *S. typhimurium* chromosome include proteins which are homologous to components of type III protein secretion systems of other bacteria (10). Several protein targets of the *Salmonella* type III secretion system have been identified which appear to play a role in invasion of cultured cells (5, 20, 27, 37). Although the *inv* locus is essential for invasion of cultured cells, the in vivo significance of this locus is less clear. The aim of the present study was to investigate the importance, in vivo, of the *inv* locus during the early stages of *Salmonella* infection. We have compared the abilities of strains carrying mutations in *invA* or *invG* to invade cultured polarized epithelial Madin-Darby canine kidney (MDCK) cells and murine Peyer's patch M cells with those of their parent strains.

S. typhimurium strains SL1344 (provided by C. L. Francis, Stanford University, Stanford, California), SR11 and its isogenic nonpolar *invA* mutant SB111 (30) (provided by J. E. Galán, State University of New York at Stonybrook), and TNP-5 (a derivative of TML) and its isogenic mutant 83, which has a Tn*phoA* insertion in *invG* (29), were grown as previously described (3). Each strain was diluted in modified Krebs buffer (22) and added to the apical compartment of MDCK II cells cultured on permeable supports (0.5-cm^2 Anocell; Nunc, Roskilde, Denmark) as described previously (4) to give a multiplicity of infection of approximately 20 bacteria per cell. The monolayers were then maintained at 37°C in air for 60 min. Cell monolayers on filter units were washed thoroughly in phosphate-buffered saline (PBS) to remove nonadherent bacteria and transferred to PBS at 0°C to prevent further invasion. *S. typhimurium* invasion and adherence were then quantified as described previously (23). Briefly, adherent bacteria were localized with anti-*Salmonella* antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies prior to cell permeabilization with methanol and incubation again with anti-*Salmonella* antibodies followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies to label all cell-associated bacteria (i.e., bacteria that had invaded or adhered).

The *invA* and *invG* mutants exhibited markedly attenuated

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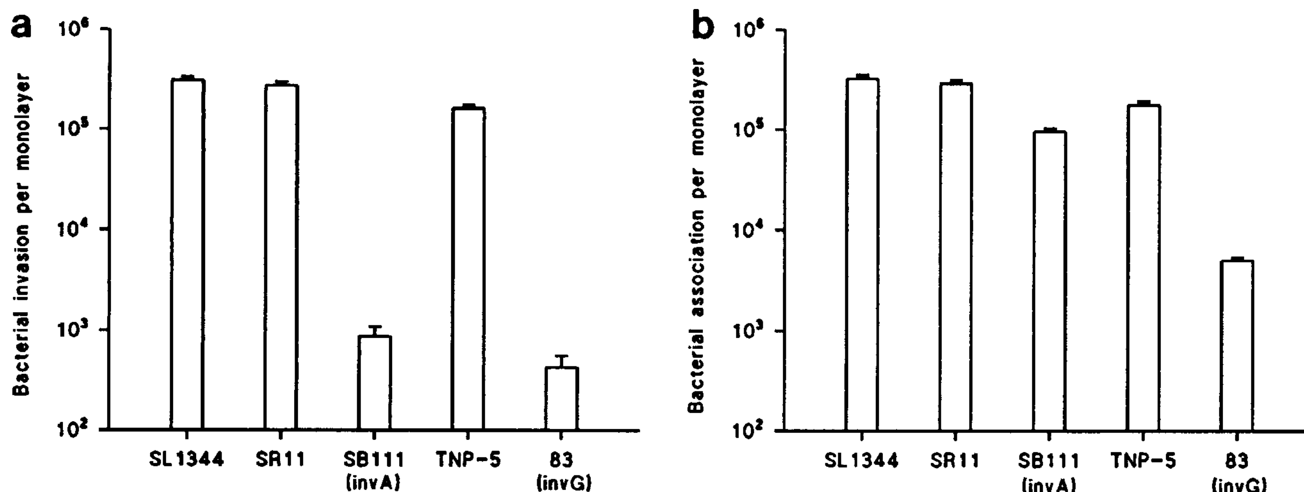

FIG. 1. (a) Invasion (log scale) of MDCK cell monolayers by *S. typhimurium* strains after 60 min of infection at a multiplicity of infection of 20. Parental strains SR11 and TNP-5 invade at levels equivalent to those of the alternative wild-type SL1344, while *invA* and *invG* mutants (SB111 and 83) are attenuated by ca. 300-fold compared with their respective parents. Data are expressed as means ± standard errors of the means (n = 5 to 6). (b) Association (adherence plus invasion) of *S. typhimurium* strains with MDCK cell monolayers from the same experiment as that from which were generated the data in panel a (log scale). Note the small decrease in cell association observed for *invA* mutant SB111, indicating that adherence is not significantly affected by this mutation. The ca. 30-fold decrease in the number of mutant 83 bacteria associated with the monolayers indicates that this *invG* mutation affects bacterial adherence under these conditions. Data are expressed as means ± standard errors of the means (n = 5 to 6).

invasion of MDCK cells compared with their parent strains and the alternative wild-type strain SL1344 (Fig. 1). The *invA* mutant SB111 exhibited 0.3% of the level of invasion of the wild-type strain SR11 ($P < 0.01$; Mann-Whitney U test), while the total number of SB111 associated with the monolayers was moderately reduced to 33.1% of that observed after infection with SR11 ($P < 0.01$; Mann-Whitney U test) (Fig. 1). The *invG* mutant 83 was also markedly deficient for invasion of MDCK cells, exhibiting 0.3% of the level of invasion demonstrated by parent strain TNP-5 ($P < 0.01$; Mann-Whitney U test) (Fig. 1a). However, association of mutant 83 with monolayers was significantly attenuated (to 2.8%) compared with that of TNP-5 ($P < 0.01$; Mann-Whitney U test) (Fig. 1b), suggesting a role for *invG* in cell adherence which was not previously observed with an alternative *invG* mutant in Henle-407 cells (26).

The markedly attenuated invasion of polarized MDCK cells by *invA* and *invG* mutants measured by the immunocytochemical procedure (Fig. 1) confirms data obtained with these and similar mutants in several cell lines, including MDCK, by using invasion assays based on sensitivity to gentamicin (12, 13, 17, 21, 26, 29). The lack of invasion of MDCK cells by *invA* and *invG* mutants also correlates with the inability of these mutants to induce membrane ruffling in these cells as determined by scanning electron microscopy (SEM) (17, 21, 23).

Ligated jejunal-ileal gut segments containing Peyer's patches were created in anesthetized adult female BALB/c mice and infected, as described previously (3), with *S. typhimurium* strains which had been grown as described above; they were then pelleted, washed twice in PBS, and resuspended in PBS to give total counts of 3×10^9 bacteria per ml. The gut loops were harvested after 15, 30, 60, or 120 min, and the mice were culled by cervical dislocation. Harvested tissues were fixed and processed for cytochemical localization of *Salmonella* cells with specific antibodies (3) and of M cells with the selective lectin *Ulex europaeus* 1 (UEA1) (2) and/or for SEM (2, 3).

Examination of UEA1-stained Peyer's patch tissues revealed that following incubation in mouse gut loops, the distribution of both sets of parent and mutant bacteria was similar to that documented previously for the mouse-virulent *S. typhimurium* SL1344 (3). The vast majority of cell-associated bacteria were restricted to the FAE. Few bacteria were clearly associated with villous epithelia, although large numbers were located in mucus (where present) above FAE and villi. Within the FAE, the majority of bacteria were associated with M cells, bacteria being more numerous at the periphery of the domes, where M cells are concentrated. The bacteria were not, however, distributed evenly on the M cells. Most salmonellae were associated with small groups of M cells; the latter were dispersed within regions of FAE containing M cells with few or no associated bacteria. Bacteria associated with the apical regions of M cells were frequently surrounded by regions of intense UEA1 staining observed as distinct rings on confocal optical sections (Fig. 2a).

Confocal laser scanning microscopy (CLSM) revealed that the parent strains (TNP-5 and SR11; data not shown) and their isogenic mutants, 83 and SB111 (Fig. 2), adhered to and invaded mouse Peyer's patch M cells. There was no apparent difference between *invA* and *invG* mutants and their parent strains with respect to their interaction with M cells. Pronounced variability of bacterial association between individual lymphoid follicles and regional variation within single follicles rendered quantification impracticable, although it was clear that each strain exhibited significant invasion of M cells. Association of bacteria with M cells increased with duration of infection, although some bacteria that had invaded or adhered were observed after only 15 min. After longer incubation periods (60 to 120 min), bacteria (both parent and mutant strains) were frequently located at least 20 μm below the apical surface of the cells, where it was not possible to reliably determine which cells had initially been invaded.

SEM examination of infected Peyer's patches revealed that both parent and mutant bacteria induced remodelling of the apical membrane of mouse Peyer's patch M cells to form prominent membrane extrusions which were heterogeneous in form but often closely resembled the structures termed membrane ruffles that had previously been observed in association with wild-type *S. typhimurium*. The frequency and distribution

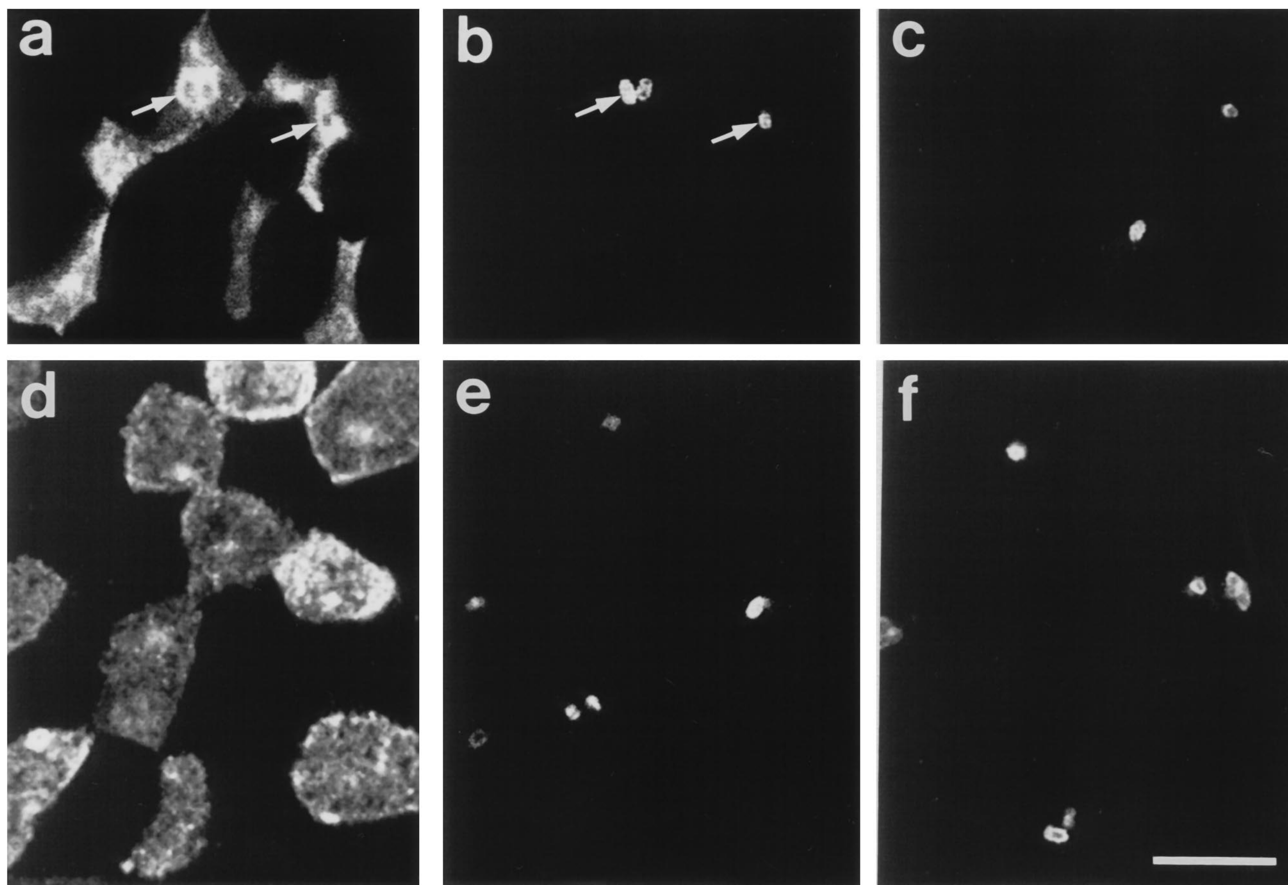

FIG. 2. CLSM views of FAE from murine Peyer's patch gut loops incubated with *invA* mutant SB111 (a to c) for 60 min or *invG* mutant 83 (d to f) for 120 min. (a and d) Surface views of FITC-stained M cells; (b and e) TRITC-stained bacteria on M-cell surfaces; (c and f) TRITC-stained bacteria at 3 and 4 μm, respectively, below the M-cell surfaces. Bacterial adhesion to M cells in panel a is accompanied by a redistribution of UEA1 staining around the adherent bacteria (arrows). Redistribution of UEA1 binding sites was observed around the sites of interaction of some parent, *invA*, and *invG* mutant *Salmonella* strains. Bar, 10 μm.

of these areas of membrane remodelling were consistent with bacterial invasion observed by CLSM. This correlation was confirmed by SEM examination of regions of FAE which had previously been stained to reveal M cells and salmonellae and imaged by CLSM (Fig. 3 and 4). Mutations in *invA* and *invG* had no apparent effect on membrane remodelling. In addition to the extensive membrane remodelling observed on M cells, smaller membrane extrusions were infrequently observed on FAE enterocytes. Regions of gross epithelial disruption or M-cell destruction which have previously been documented in gut loops infected with wild-type *S. typhimurium* by an alternative protocol (25) were never observed by SEM in the present study, suggesting that this previously described phenomenon may be dependent on the infection protocol employed.

The fact that the *invA* and *invG* mutants readily invaded mouse Peyer's patch M cells in vivo demonstrates that the type III protein secretion system encoded by the *inv* locus and neighboring regions of the *S. typhimurium* chromosome is not the sole determinant of intestinal invasion. Of course, these data do not rule out the possibility that the *inv* locus does contribute to intestinal invasion in vivo, as is suggested by the finding that *invA* mutants exhibit reduced virulence in mice when administered orally but not when given intraperitoneally (11). Invasion of M cells by each *inv* mutant was accompanied by extensive remodelling of the apical membrane, which was indistinguishable from that induced by the parent strains. It is clear that *Salmonella* uptake by M cells differs from that of inert particles, such as polystyrene microspheres, which are also actively taken up by M cells (24, 31, 33) as it involves the triggering of cell responses which are not observed during the uptake of inert particles (24, 33).

Previously, the lack of invasion by nonpolar *invA* mutants, such as SB111, in cultured-cell models had been correlated with the failure of these mutants to induce membrane ruffling (13, 17, 23), aggregation of surface proteins (14), and intracellular signal transduction events (13, 30). Here, we show that M-cell invasion by *invA* mutant SB111 is associated with membrane rearrangements indistinguishable from those induced by the parent strain, SR11, and closely resembling those observed in cultured cells by ourselves and others. Furthermore, surface UEA1 binding sites were clustered around the site of bacterial internalization into M cells, which is reminiscent of the aggregation of surface proteins associated with *S. typhimurium*-induced membrane ruffling of HeLa cells (14). Our work shows that invasion of M cells and associated membrane remodelling can proceed independently of the *inv*-mediated mechanisms demonstrated in cultured cells.

Polar *invA S. typhimurium* mutants were previously reported to exhibit approximately 50-fold increases in 50% lethal dose values and a somewhat reduced (ca. 8-fold) capacity to colonize Peyer's patches after oral administration in mice (11).

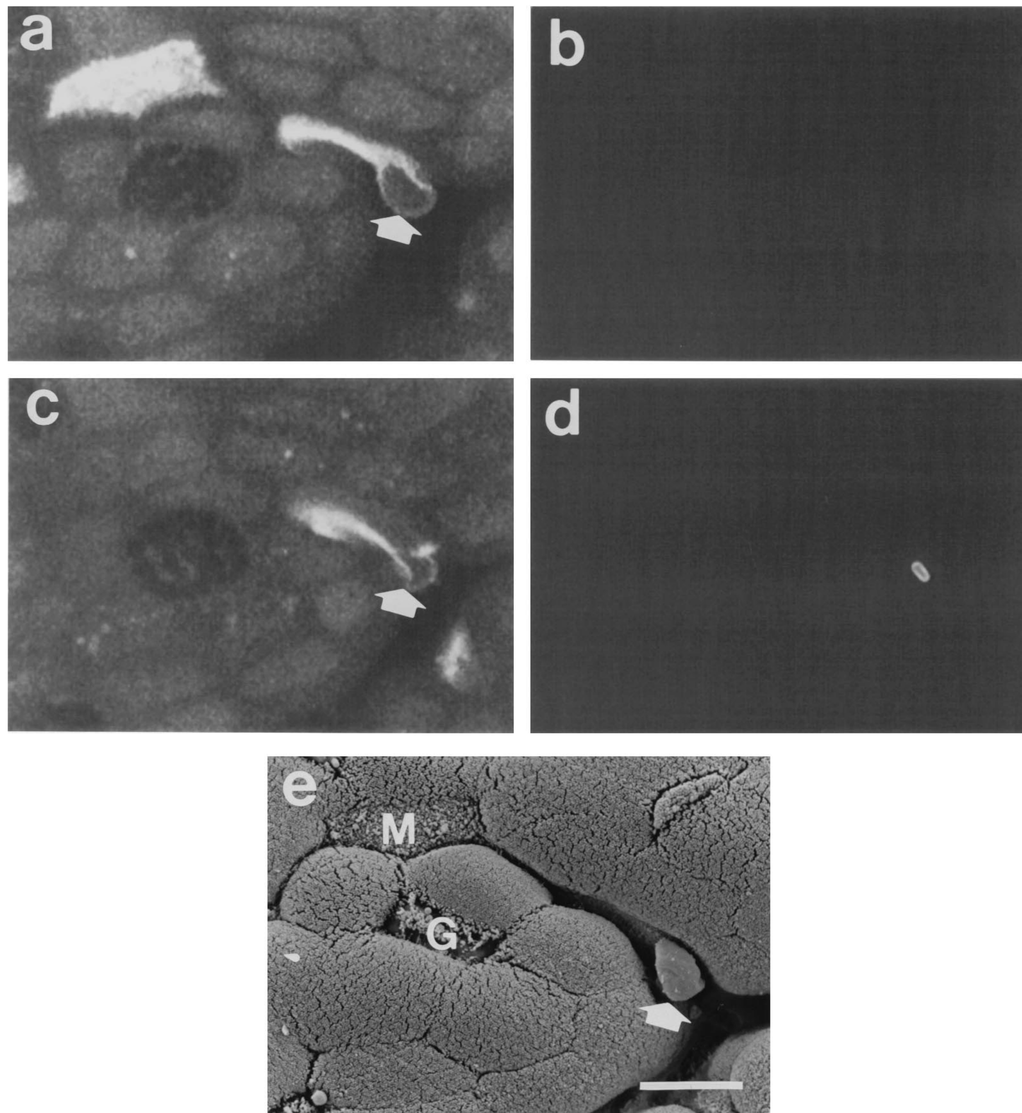

FIG. 3. CLSM (a to d) and SEM (e) images of a single region of mouse Peyer's patch FAE obtained from a gut loop incubated in vivo for 60 min with *S. typhimurium invA* mutant SB111. CLSM images (surface [a and b] or at a depth of 2 μm [c and d]) depict tissue dual stained for M cells (TRITC labelled [a and c]) and *Salmonella* cells (FITC labelled [b and d]). *Salmonella* cells are absent from the FAE surface, but panel d shows a single bacterium within the M cell (indicated by arrows in panels a and c) which, as SEM (panel e) reveals, displays marked alteration of apical membrane morphology (arrow). Also observed in this region of FAE are an M cell displaying normal microvillus morphology (M), a goblet cell (G), and enterocytes (unlabelled) displaying normal morphology. Bar, 5 μm.

While these mutants were of reduced virulence, they still killed mice. This may be explained by our finding that nonpolar *invA* mutants retain their ability to invade M cells. Although our observation that the *invA* mutant SB111 readily invades murine M cells indicates that previous reports may have exaggerated the importance of this genetic locus in intestinal invasion, we cannot exclude the possibility that a relatively minor attenuation of invasiveness might not be detected by our microscopic analyses or that the infection conditions employed did not enable operation of a separate, *inv*-mediated invasion mechanism. It has previously been reported that a polar *invA* mutant failed to induce epithelial damage characteristic of wild-type *S. typhimurium* in a murine gut loop model (25). In our studies, neither parental nor *inv* mutant strains induced gross epithelial damage. Further studies will be required to explain this difference.

We have also demonstrated that M cells are readily invaded by *S. typhimurium* carrying a mutation in *invG* (mutant 83). This gene encodes a protein translocase homologous to components of the type III protein secretion apparatus of other bacteria (26), which has been shown by our data and that of others (26, 29) to be necessary for invasion of cultured cells. Neighboring gene clusters *spa* and *prgHIJK* have also been implicated in both protein secretion and invasion of epithelial cells (18, 20, 32). Several targets of the *Salmonella inv*-encoded protein export system have recently been identified which are required for invasion of cultured cells (5, 20, 27, 32, 37). The *inv*-encoded protein export system appears not to be the sole determinant of M-cell invasion, since the *invG* mutant 83 readily invaded these cells. Although this suggests that the recently described *Salmonella* secreted proteins are also not required to trigger M-cell invasion, we cannot at present discount the possible involvement of an alternative type III protein secretion system, such as that implied by the recent description of additional *S. typhimurium* virulence genes homologous to *invG* and *invA* (19, 34).

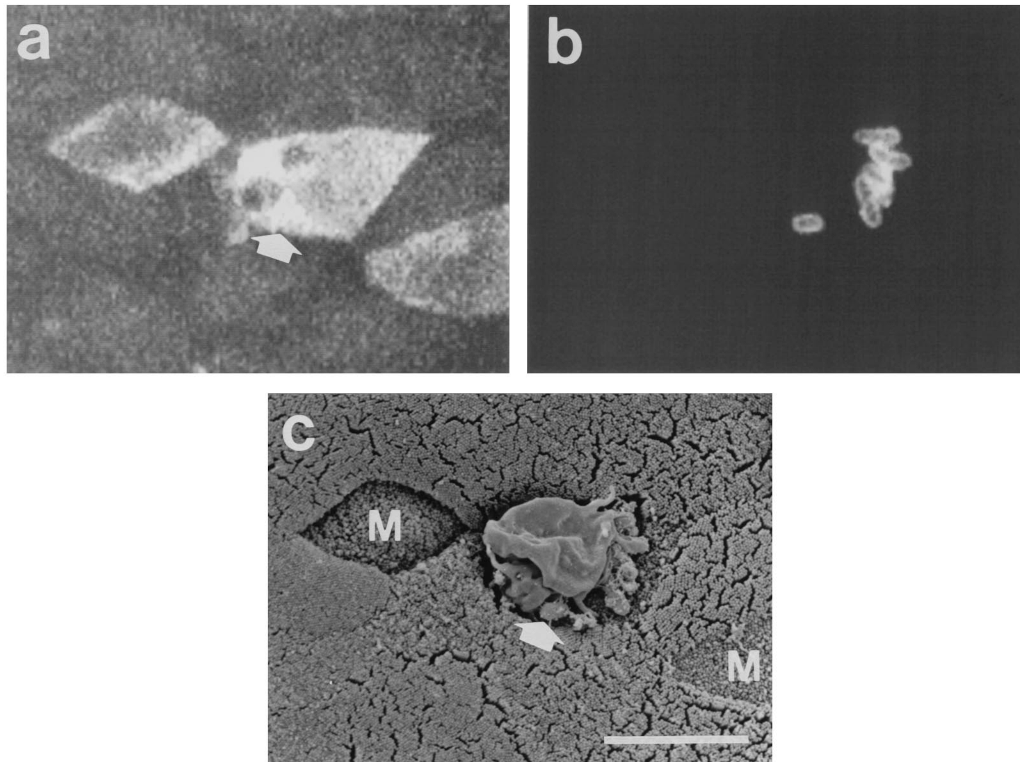

FIG. 4. CLSM (a and b; projected series) and SEM (c) images of a single region of mouse Peyer's patch FAE obtained from a gut loop incubated in vivo for 60 min with *S. typhimurium invG* mutant 83. CLSM images depict TRITC-stained M cells (a) and FITC-stained *Salmonella* cells (b). The bacteria in panel b are associated with an M cell (arrow, panel a) displaying prominent remodelling of the apical membrane resembling membrane ruffling (arrow, panel c). Also observed in this region are enterocytes (unlabelled) and two M cells (M) displaying normal microvillus morphology. Bar, 5 μm.

Our observation that *invA* and *invG* mutants retain their ability to invade M cells demonstrates that alternative or additional entry pathways operate in vivo which may be related to the unique properties of the specialized antigen-transporting M cells. The demonstration of *inv*-independent intestinal invasion supports previous reports that in vitro invasion assays do not always correlate with in vivo invasion. For example, Lodge et al. (29) reported that *invG*, *invH*, and *pagC* mutants of *S. typhimurium* were hypoinvasive in HEp-2 cells but invaded rabbit intestine as readily as their parental strains in an organ culture model. These data demonstrate that the extent and mechanisms of *Salmonella* invasion are dependent on cell type and are consistent with the previous suggestion that alternative *Salmonella*-induced signalling pathways may operate in different cell lines (9). The multifactorial nature of *Salmonella* invasiveness may contribute to its broad host specificity and its ability to invade a wide variety of cell types during systemic infection.

In conclusion, the present study has demonstrated that active invasion of murine M cells by *S. typhimurium* may proceed via mechanisms independent of the *inv* locus. These data emphasize the complexity of the genetic basis for *Salmonella* internalization and suggest that a combination of in vitro and in vivo techniques will be necessary to unravel the diverse cellular interactions involved in *Salmonella* pathogenesis.

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