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Exploitation of microfilament proteins by *Listeria monocytogenes*: microvillus-like composition of the comet tails and vectorial spreading in polarized epithelial sheets

Constance J. Temm-Grove[1,*], Brigitte M. Jockusch[1], Manfred Rohde[2], Kirsten Niebuhr[2], Trinad Chakraborty[3] and Jürgen Wehland[2,†]

[1]Cell Biology, Zoological Institute, Technical University Braunschweig, D-38106 Braunschweig, Germany
[2]Cell Biology/Immunology and Microbiology, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany
[3]Institute for Medical Microbiology, University of Giessen, D-35392 Giessen, Germany

*Present address: Cold Spring Harbor Laboratories, Demerec Laboratory, Cold Spring Harbor, NY 11724, USA
†Author for correspondence

SUMMARY

Effective cell-to-cell spreading of the facultative intracellular pathogen *Listeria monocytogenes* requires the interaction between bacteria and the microfilament system of the host cell. By recruiting actin filaments into a 'comet tail' localized at one pole of the bacterial cell wall, *Listeria* become mobile and propel themselves through the cytoplasm. They create protrusions at the plasma membrane that can invaginate adjacent cells. In this work, we have analysed the structural composition of *Listeria*-recruited microfilaments in various epithelial cell lines by immunofluorescence microscopy. The microfilament-crosslinking proteins alpha-actinin, fimbrin and villin were localized around bacteria as soon as actin filaments could be detected on the bacterial surface. Surprisingly, the same was found for ezrin/radixin, proteins involved in linking microfilaments to the plasma membrane. We found that in a polarized cell line derived from brush border kidney epithelium (LLC-PK1), the actin filaments surrounding intracytoplasmic motile bacteria show the same immunoreactivity as the brush border-like microvilli, when analysed by a specific actin antibody.

The successful invasion of polarized LLC-PK1 islets is vectorial, i.e. it progresses predominantly from the periphery of the islets towards the centre. Infection of the peripheral cells is sufficient for infiltration of the entire cellular islets, without any further contact with the extracellular milieu. This is in contrast to nonpolarized epithelial sheets, which can be invaded from the apical surface of any individual cell. The importance of active bacterial motility in this vectorial spreading is emphasized by our finding that an isogenic *Listeria* mutant that is unable to recruit actin filaments cannot colonize polarized epithelial layers but accumulates in the peripheral cells of the islets.

Key words: cytoskeleton, actin filament, polarized epithelia, intracellular motility, intracellular parasite

INTRODUCTION

Recently, the mode of locomotion of intracellular parasitic bacteria has gained much interest as a model system in which to study microfilament assembly and function (see Tilney and Tilney, 1993). In the case of *Listeria*, a food-borne bacterial pathogen that can cause severe systemic infections in susceptible individuals, the initial site of bacterial entry into the host is presumably the intestine. There, the precise locus of invasion is not known, but during an acute infection, many tissues are infected, demonstrating the ability of *Listeria* to invade numerous eukaryotic cell types (Gellin and Broome, 1989). In 1970 elegant electron microscopical studies showed that *Listeria monocytogenes* invades epithelial cells of the cornea (Racz et al., 1970). Moreover, *Listeria* were detectable within intestinal epithelial cells in orally infected animals (Racz et al., 1972) and, using tissue culture invasion assays, *Listeria* have been shown to be capable of penetrating various cell types, including hepatocytes and fibroblasts (see Cossart and Mengaud, 1989). Once inside a phagosome or an analogous compartment, the bacteria actively dissolve the vacuolar membrane by secreting a haemolysin (listeriolysin) and proliferate subsequently within the cytoplasm of the host cell (Gaillard et al., 1987). The bacteria then induce the polymerization of short actin filaments, which are subsequently organized into a 'comet tail', located at one pole of their surface (Tilney and Portnoy, 1989; Mounier et al., 1990). The characterization of *Listeria* mutants unable to accumulate actin filaments led to the identification of the bacterial ActA polypeptide, which is essential (Domann et al., 1992; Kocks et al., 1992) and sufficient (Pistor et al., 1994) for initiation of bacterially induced actin filament assembly.

Previous studies have shown that the actin filaments initially surrounding intracellular bacteria as well as those in the fully

grown tails contain several actin binding proteins (Dabiri et al., 1990; Sanger et al., 1992). Ultrastructural analyses have revealed that these microfilaments are short (up to 0.2 μm) and crosslinked with each other and the surrounding cytoskeleton (Tilney and Portnoy, 1989; Tilney et al., 1992a,b). In the comet tail, such structures probably provide a temporary platform for newly polymerizing actin filaments, and the polymerization process itself is thought to be the basis for bacterial motility, as the rate of polymerization correlates with the velocity of bacterial movement (Sanger et al., 1992; Theriot et al., 1992). The rapid propulsion through the cytoplasm results in frequent contact with the inner face of the plasma membrane of the infected cell, leading to the formation of finger-like protrusions each with a bacterium at its tip. Such a protrusion can invaginate an adjacent cell, resulting in its phagocytosis. This provides an effective mechanism to spread the infection (Tilney and Portnoy, 1989; Mounier et al., 1990). However, while this view on motility and spreading of *Listeria* is generally accepted, the details of the precise structural composition, the functions of the various components and the dynamics of the microfilament structures recruited by the parasite are still unclear.

In this study, we have analysed the protein composition of the bacterial motile machinery, as recruited in infected epitheloid cells. Our data indicate that actin is assembled into filaments that share certain conformational features with the microvillar actin core and is complexed with several proteins that are normally found in specific cytoskeletal structures, such as microvilli and the cortical actin network. In addition, we demonstrate that invasion of cultured epithelial cells of the brush border type takes place from the periphery of a colony in a vectorial fashion.

MATERIALS AND METHODS

Bacterial strains, media and reagents

The weakly haemolytic *L. monocytogenes* strain EGD (serotype 1/2a) and its isogenic *act*A1 mutant have been described previously (Domann et al., 1992). *Listeria* were grown in brain/heart infusion broth (Difco) at 37°C, with 5 μg erythromycin per ml in the case of the *act*A1 mutant. All chemical reagents were purchased from Sigma (Deisenhofen) unless indicated otherwise.

Tissue culture, infection and immunofluorescence microscopy

PtK_2 cells (ATCC CCL56), and the pig kidney epithelial cell lines LLC-PK1 (ATCC CCL101) and PK_{15} (ATCC CCL33), were raised in minimum essential medium (MEM, Gibco) supplemented with 10 % fetal calf serum, glutamine and nonessential amino acids in the absence of antibiotics. For infection experiments, cells were grown on 12 mm coverslips and infections with the *L. monocytogenes* wildtype EGD and the mutant strain were performed as previously described (Niebuhr et al., 1993). One hour after starting the infection the cells were washed up to 5 times with fresh complete medium supplemented with 25 μg of gentamycin per ml and incubated for up to 6 hours in the same medium. For analysing vectorial spreading of *L. monocytogenes* in the LLC-PK1 cell line, the cells were seeded at low density on glass coverslips in 6-well plates and grown for 4 days to obtain proper differentiated islets and cellular sheets that revealed apical microvilli as judged by scanning electron microscopy (SEM). After adding the bacteria the plates were centrifuged at 1,000 ***g*** for 3 minutes, incubated for 30 minutes, washed extensively with fresh medium, then fixed immediately or incubated for further 30 minutes in fresh medium before adding gentamycin and incubating for various periods. Treatment of noninfected differentiated LLC-PK1 cells with EGTA prior to infection was carried out essentially as described by Mounier et al. (1992) for the human Caco-2 epithelial cell line. The coverslips were then rinsed with PBS, and the cells were either fixed in 3.7% formaldehyde in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS or with cold (−20°C) methanol or processed for SEM. Alternatively, the cells were fixed and permeabilized with methanol at −20°C for 6 minutes or with ethanol at room temperature for 30 seconds. Coverslips were incubated with fluorescein- or rhodamine-labelled phalloidin mixed with the primary polyclonal or monoclonal antibodies followed by rhodamine- or fluorescein-labelled secondary antibodies (Dianova) and further processed as described previously (Niebuhr et al., 1993). Samples were examined with a Zeiss Axiophot microscope equipped with epifluorescence. Photographs were taken with Kodak Tri-X films.

Antibodies

The affinity-purified chicken antibodies against rabbit muscle actin (Schrader et al., 1994) and the polyclonal antibodies against formaldehyde-fixed *Listeria* (Domann et al., 1992) have been described previously. Monoclonal alpha-actinin antibodies (A 5044) were purchased from Sigma, monoclonal villin antibodies were from Dianova. Polyclonal antibodies against alpha-actinin and villin have been described previously (Temm-Grove et al., 1992). The affinity-purified polyclonal antibodies against fimbrin isolated from porcine intestinal brush border cells were obtained from Dr Volker Gerke (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany); the monoclonal antibody against chicken brain ezrin/radixin (13H9) was supplied by Dr Frank Solomon (MIT, Boston, USA). This antibody reacts with purified intestinal ezrin (Birgbauer and Solomon, 1989), presumably with an epitope present in ezrin as well as in radixin (Winckler et al., 1994).

Scanning electron microscopy

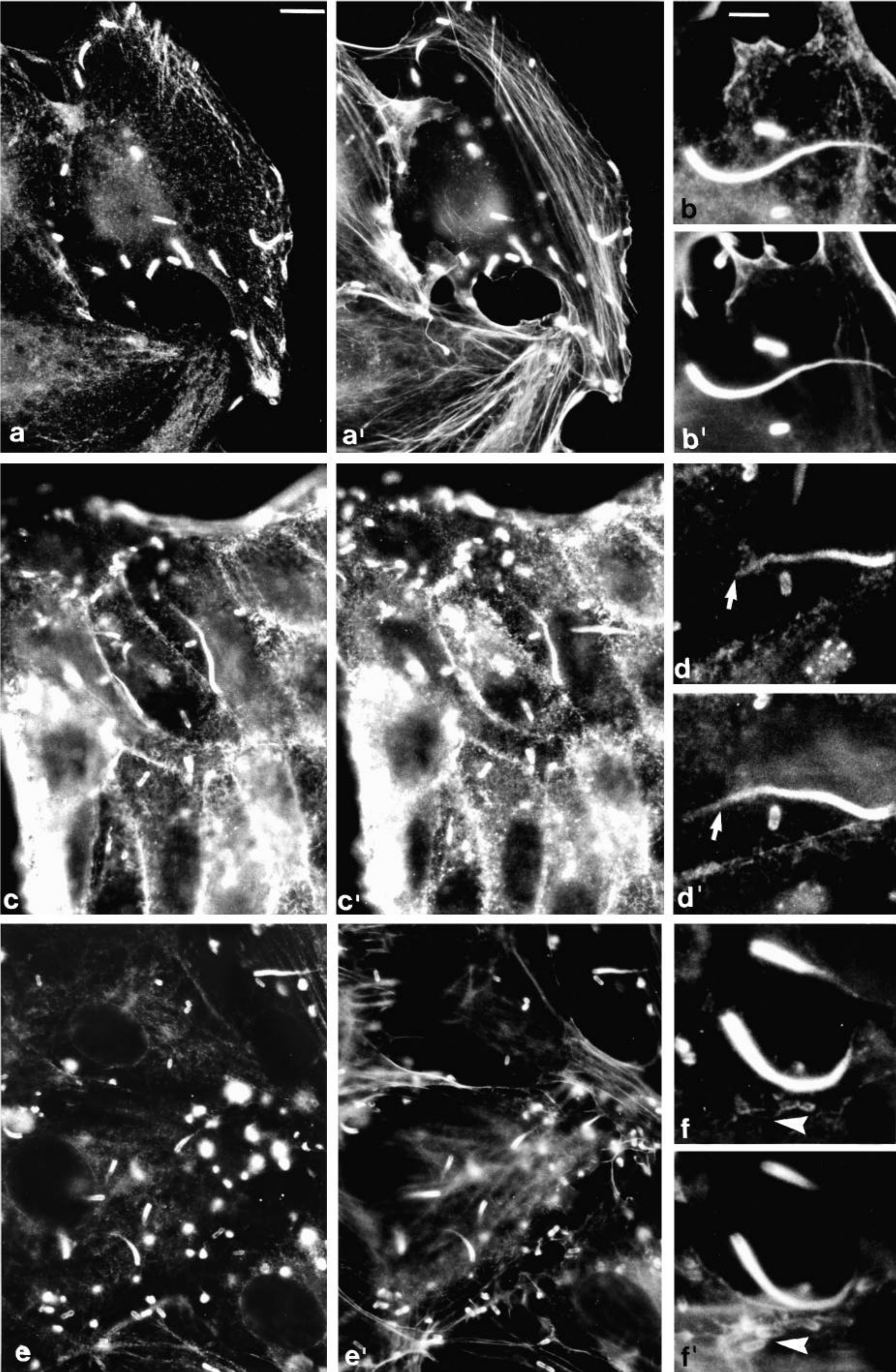
The samples were fixed in a solution containing 3% glutaraldehyde (v/v) and 5% formaldehyde (v/v) in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$) for 1 hour on ice, washed with cacodylate buffer, and dehydrated using a graded series of acetone. Subsequently, samples were critical-point-dried using CO_2, sputter-coated with gold (10 nm), and examined in a Zeiss DSM 940 scanning electron microscope at an acceleration voltage of 10 kV and calibrated magnifications.

RESULTS

Microfilament bundling proteins are associated with bacteria already early during cytoskeletal recruitment

In order to analyse the structural composition of the microfil-

Fig. 1. Codistribution of microfilament proteins with F-actin in *L. monocytogenes*-infected epithelial cell lines as seen in double-fluorescence images. (a,b) PK_{15} cells, labelled with anti-alpha-actinin/rhodamine. (c,d) LLC-PK1 cells labelled with anti-fimbrin/rhodamine. (e,f) PK_{15} cells, labelled with anti-ezrin/radixin/ rhodamine. (a′-f′) F-actin labelling with FITC-phalloidin. The arrow in d,d′ points to a bacterium with a very long F-actin tail (d′) that is not labelled with anti-fimbrin at the most distal region. The arrowhead in f, f′ marks bacteria that already possess an F-actin coat (f′) but are negative for ezrin/radixin labelling (f). Bars: (a) valid for (a,a′,c,c′,e,e′), 15 μm; (b) valid for (b, b′, d, d′, f, f′), 5 μm.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ament elements recruited by *L. monocytogenes* and to study the mode of infection in different epitheloid cell types, we infected several cell lines of epithelial origin with this parasite and followed intracellular events using fluorescence microscopy. We studied three different lines of kidney origin: PtK_2 cells, a cell line that is widely used for studying *Listeria* infection (Sanger et al., 1992; Theriot et al., 1992; Niebuhr et al., 1993), PK_{15} and LLC-PK1 cells. Of these, only the latter one is capable of reconstituting a polarized epithelium-like architecture in culture, which requires the synthesis of villin and assembly of microvilli of the brush border type (Temm-Grove et al., 1992). The infected cells were processed for double-fluorescence microscopy, using fluorescently labelled phalloidin for actin localization and specific antibodies for various microfilament-associated proteins. The results of these studies are presented in Fig. 1. In agreement with previous studies (Dabiri et al., 1990; Sanger et al., 1992), immunolabelling of *Listeria*-infected PK_{15} cells revealed that alpha-actinin colocalized with actin filaments not only in the polar tail-like structures but also with the coats surrounding the intracellular bacteria, suggesting an early and rapid incorporation of alpha-actinin into the *Listeria*-induced actin filaments (Fig. 1a,a′ and b,b′). A similar pattern of colocalization with actin was seen for fimbrin in *Listeria*-infected LLC-PK1 cells. In addition to its normal distribution in various types of cellular projections, including lamellopodia and microvilli, this protein was clearly detectable around intracellular bacteria and in the comet tails (Fig. 1c,c′ and d,d′). A codistribution of fimbrin with *Listeria*-recruited actin filaments has also previously been described by Kocks and Cossart (1993). Interestingly, the distal ends of some long comet tails revealed only faint or no labelling with fimbrin antibodies, suggesting that this protein is lost from older tails, and that such a release may precede the depolymerization of actin filaments in these structures (compare d with d′ in Fig. 1).

The distribution of ezrin/radixin, members of the family of highly related proteins (the ERM proteins: ezrin, radixin, moesin; cf. Arpin et al., 1994) was also analysed in *Listeria*-infected cells. Under normal conditions, these proteins are restricted to the cortical cytoskeleton and localized in cellular protrusions, such as microvilli, lamellopodia and microspikes (Bretscher, 1983, 1991; for further references, see Arpin et al. 1994). Surprisingly, in *Listeria*-infected PK_{15} cells, ezrin/radixin, as identified by the 13H9 monoclonal antibody, was found to be additionally localized around the cytoplasmic bacteria and along the entire length of the actin tails. However, we did occasionally encounter bacteria that were coated with actin but showed no clear staining for these proteins (compare f with f′ in Fig. 1).

Like fimbrin, villin is an actin filament bundling protein that is found exclusively in cellular protrusions. However, it is normally restricted to microvilli of the brush border type and is not present in other kinds of cortical extensions (Robine et al., 1985). The LLC-PK1 cell line synthesizes this protein and incorporates it into its apical microvilli (Temm-Grove et al., 1992). Following infection with *Listeria*, villin was also found to be incorporated in the actin filament layer surrounding the bacterial cell wall and in the tail structures. This was seen with both polyclonal and monoclonal anti-villin antibodies (Fig. 2).

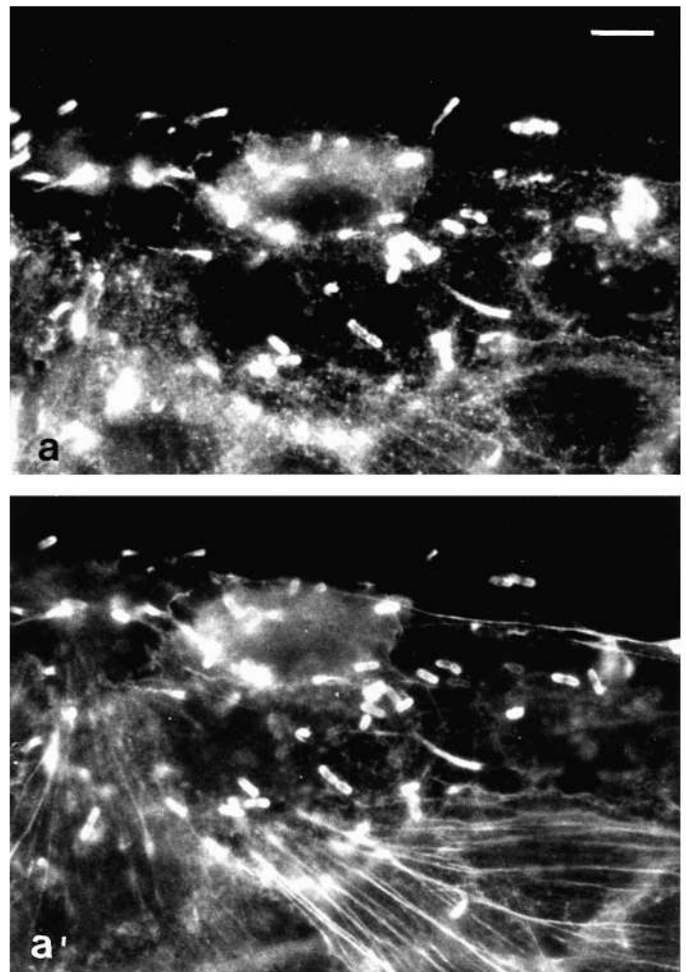


Fig. 2. The association of villin with F-actin in *L. monocytogenes*-infected LLC-PK1 cells. Double-labelling images obtained with anti-villin/rhodamine (a) and FITC-phalloidin (a′). Villin is seen as a component of the F-actin coat developed early in the infection cycle surrounding the bacterial cells, as well as of the polar ‘comet tail’ formed later in the cycle. Bar,15 μm.

Listeria-induced actin filaments share immunological properties with the microvillar core of the brush border

Previously, we have described polyclonal antibodies that were raised in chicken against a complex of DNase I and rabbit skeletal muscle actin. When affinity-purified on actin/Sepharose and analysed by immunofluorescence microscopy, these specific actin antibodies selectively recognized actin filaments in myofibrils and microvilli of the brush border-type epithelium but did not react with the other actin-containing microfilamentous structures present in nonmuscle cell types (Schrader et al., 1994). When we examined *Listeria*-infected LLC-PK1 cells with this antibody, predominantly all actin filaments recruited by bacteria were visible, i.e. those surrounding the bacteria as well as those in the tails (Fig. 3a-e). In contrast to uninfected LLC-PK1 cells (see Schrader et al., 1994), these specific actin antibodies showed hardly any staining of microvilli in heavily infected cells (see also Figs 6 and 7). The remaining cellular microfilamentous structures such as stress fibres and peripheral belts that were labelled by

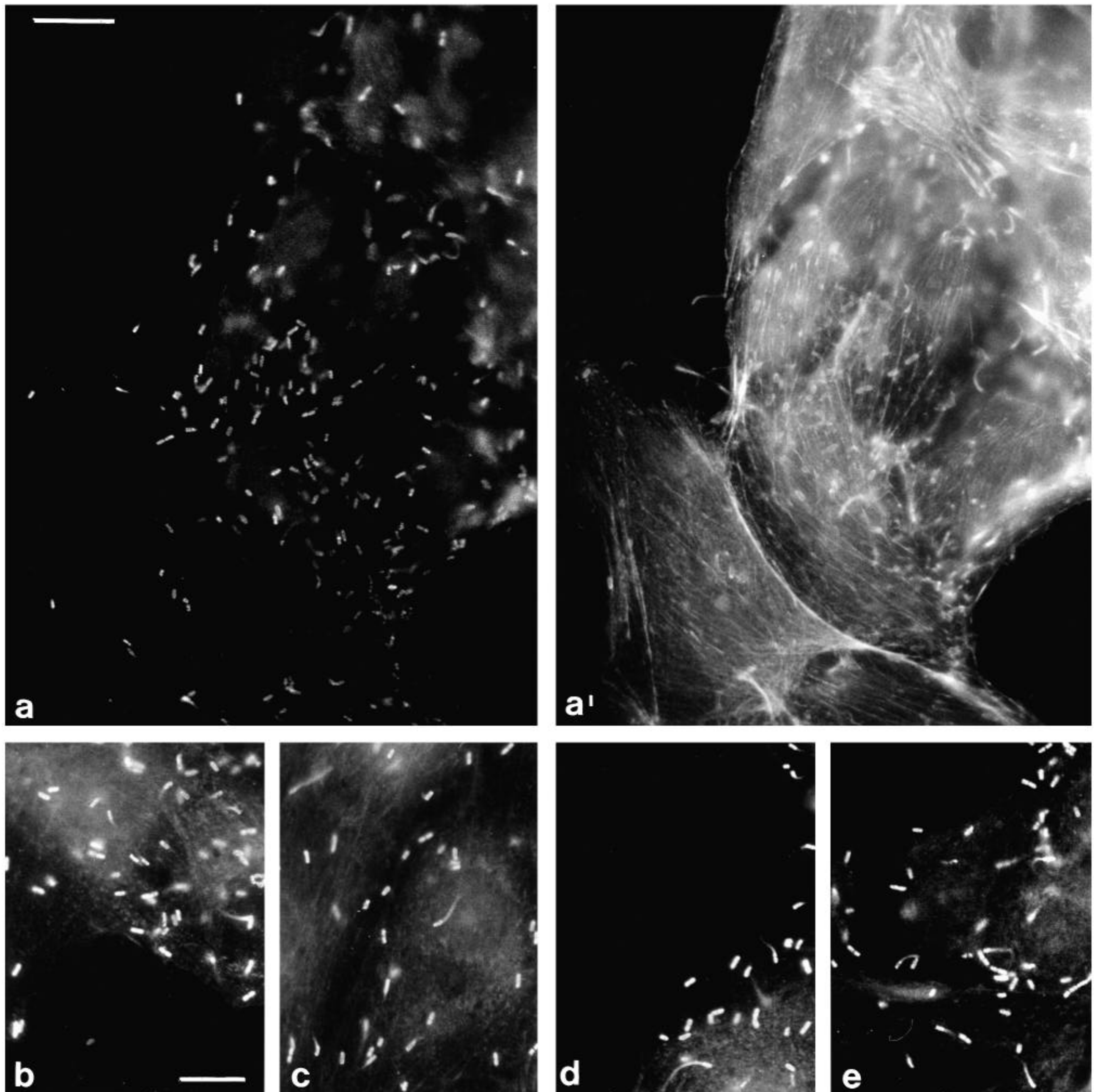


Fig. 3. Decoration of actin filaments in *L. monocytogenes*-infected LLC-PK1 cells with actin-specific reagents. (a,a′) Double-labelling with a polyclonal, affinity-purified chicken antibody against skeletal muscle actin/rhodamine (a) and FITC-phalloidin (a′). This antibody binds strongly to *Listeria*-associated actin, but not to the actin filaments in stress fibres or peripheral belts, which are seen by phalloidin staining. (b-e) Illustration of the reactivity of the chicken anti-actin with *Listeria*-induced actin coats and also with comet tails. Bars: (a) valid for (a,a′), 15 μm; (b) valid for (b-e), 15 μm.

phalloidin were not recognized by this actin antibody (Fig. 3a,a′). These findings suggest that at least in LLC-PK1 cells the actin filaments recruited in the bacterial coats as well as in the tails are arranged in a conformation similar to those in the brush border-type microvilli.

Listeria infection of polarized epithelial sheets is vectorial

Epithelial cells grown in culture usually form confluent islets containing up to several thousand individual cells. These islets can be used to study the infection and spreading of parasites entering through epithelia, such as those present in the intestinal tract. We used LLC-PK1 islets to study the mode of epithelial infection by the *L. monocytogenes* wild-type strain. At 30 minutes after starting the infection, the cells were extensively washed, either fixed or supplied with fresh medium for further 30 minutes before adding gentamycin to avoid further infection by extracellular bacteria. After various incubation periods, these islets were processed for fluorescence microscopy, using phalloidin for actin staining and a polyclonal antibody for

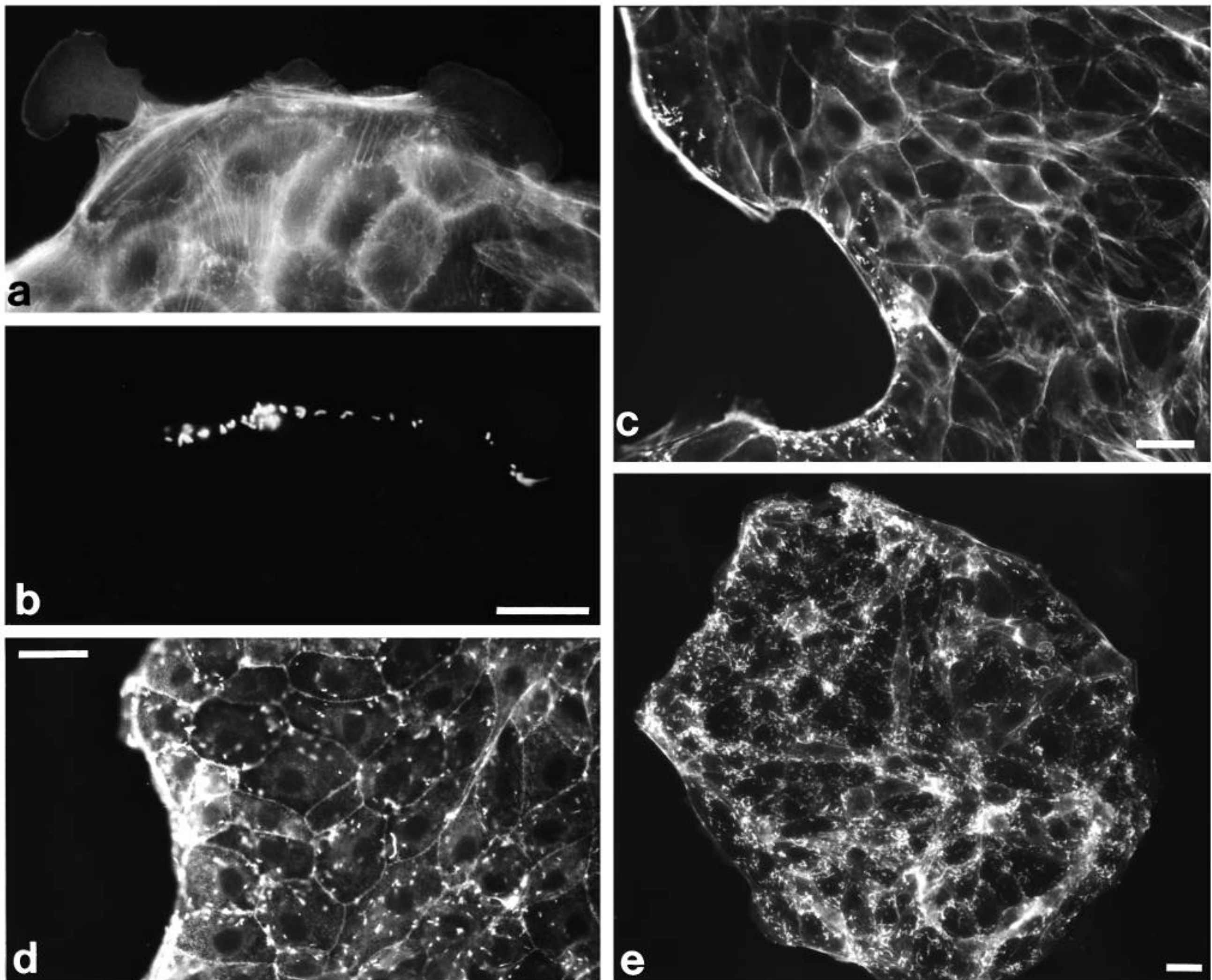


Fig. 4. Infection of LLC-PK1 cells by *L. monocytogenes* and vectorial spreading throughout epithelial sheets and islets as seen by fluorescence microscopy with FITC-phalloidin (a,c,d and e). (a,b) Peripheral cells of an islet 30 minutes after starting the infection; images were obtained by double-labelling using FITC-phalloidin (a) and polyclonal *Listeria* antibodies/rhodamine (b). After 1.5 hours peripheral cells are infected by *Listeria*, since intracelluar bacteria have accumulated actin filaments on their surface (c). (d) The periphery of an epithelial sheet after 4 hours; and (e) a small islet 6 hours after starting the infection; all cells are heavily infected. Bars, 30 μm.

detection of bacteria. As seen in Fig. 4a,b, at 30 minutes after starting the infection, bacteria were detectable at the outer edges of peripheral cells within such cellular sheets. At this early stage of infection the bacteria were not labelled by phalloidin, indicating that invasion had not occurred (compare with Fig. 6a,b) After 1.5 hours, peripheral cells were clearly infected as revealed by phalloidin staining (see Fig. 6c). At later stages, the infection progressed to the inner cells of the sheet (Fig. 4d) and, after 6 hours, smaller islets were completely colonized by *Listeria* (Fig. 4e). This could only have occurred by lateral cell-to-cell infections and must depend on active motility of the parasite. Support for this conclusion came from two control experiments. Firstly, following Ca-depletion of the tissue culture medium, bacterial infection was no longer restricted to the peripheral cells in islets or sheets, most likely due to the opening of intercellular junctions (data not shown). A similar observation has recently been made for the infection of polarized Caco-2 epithelial sheets by *Shigella flexneri* (Mounier et al., 1992). Secondly, when LLC-PK1 islets were infected with the intracellularly nonmotile *actA*1 mutant, bacteria were only detectable in the peripheral cells even after prolonged infection periods (Fig. 5). No spreading occurred and the heavily infected peripheral cells were often lysed within the first 4-5 hours after starting the infection, due to the accumulation of nonmotile bacteria within single host cells (not shown). This was never observed in sheets or islets infected with wild-type *Listeria.*

In order to analyse the different phases of bacterial entry and spreading in more detail, LLC-PK1 islets were examined by scanning electron microscopy (SEM) at different times after starting the infection (Figs 6 and 7). After 30 minutes, the majority of bacteria (more than 75%) were found attached to the outer edges of cells located at the periphery of the islets, very often at the transition between the smooth cell surface and that covered by microvilli (Fig. 6a,b). When infected islets corresponding to those shown in Fig. 4d were inspected by SEM,

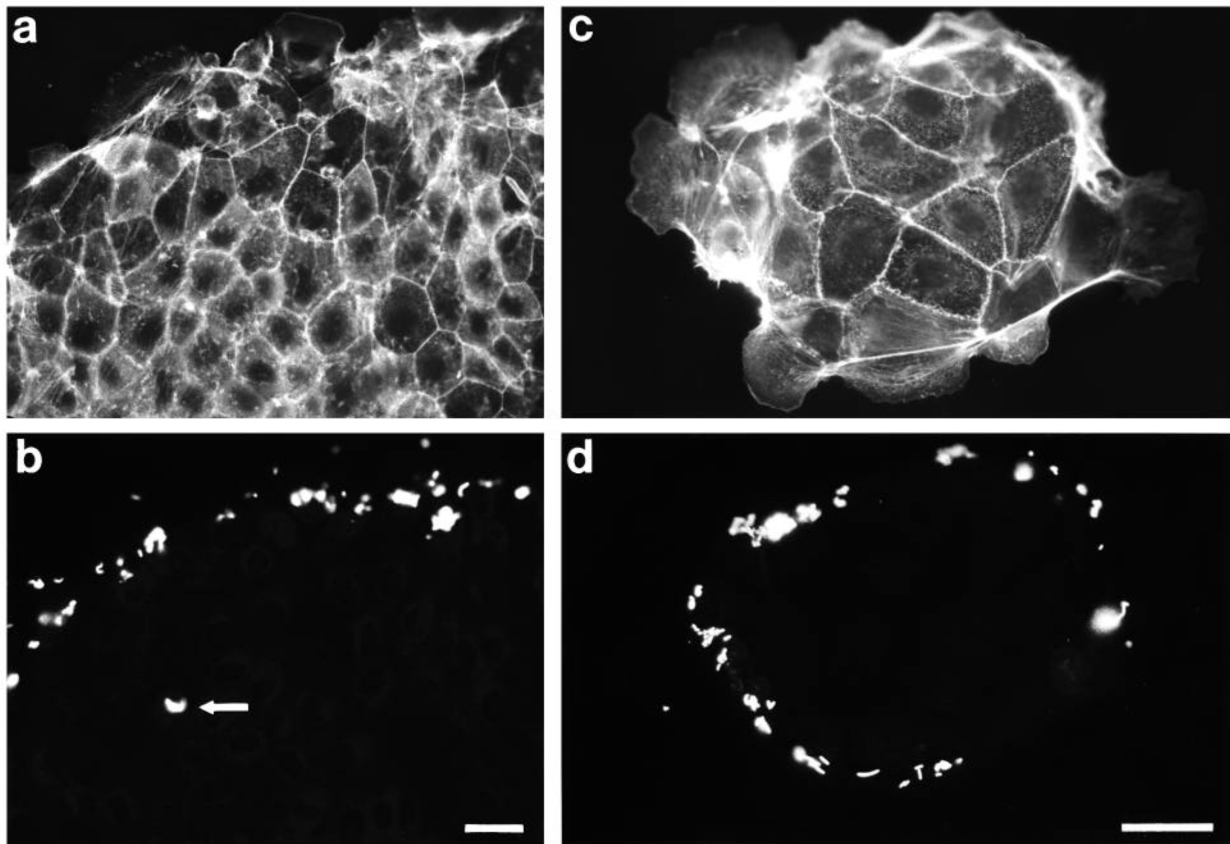


Fig. 5. Accumulation of the isogenic *act*A mutant in peripheral cells of LLC-PK1 epithelial sheets and islets. Images were obtained by double-labelling with FITC-phalloidin (a, c) and polyclonal *Listeria* antibodies/rhodamine (b,d). (a,b) Three hours after starting the infection, peripheral cells of an epithelial sheet are heavily infected with bacteria, no spreading has occurred, occasionally single cells within the sheets are infected (arrow in b). (c,d) A small islet with heavily infected peripheral cells after 3 hours. Bars, 30 µm.

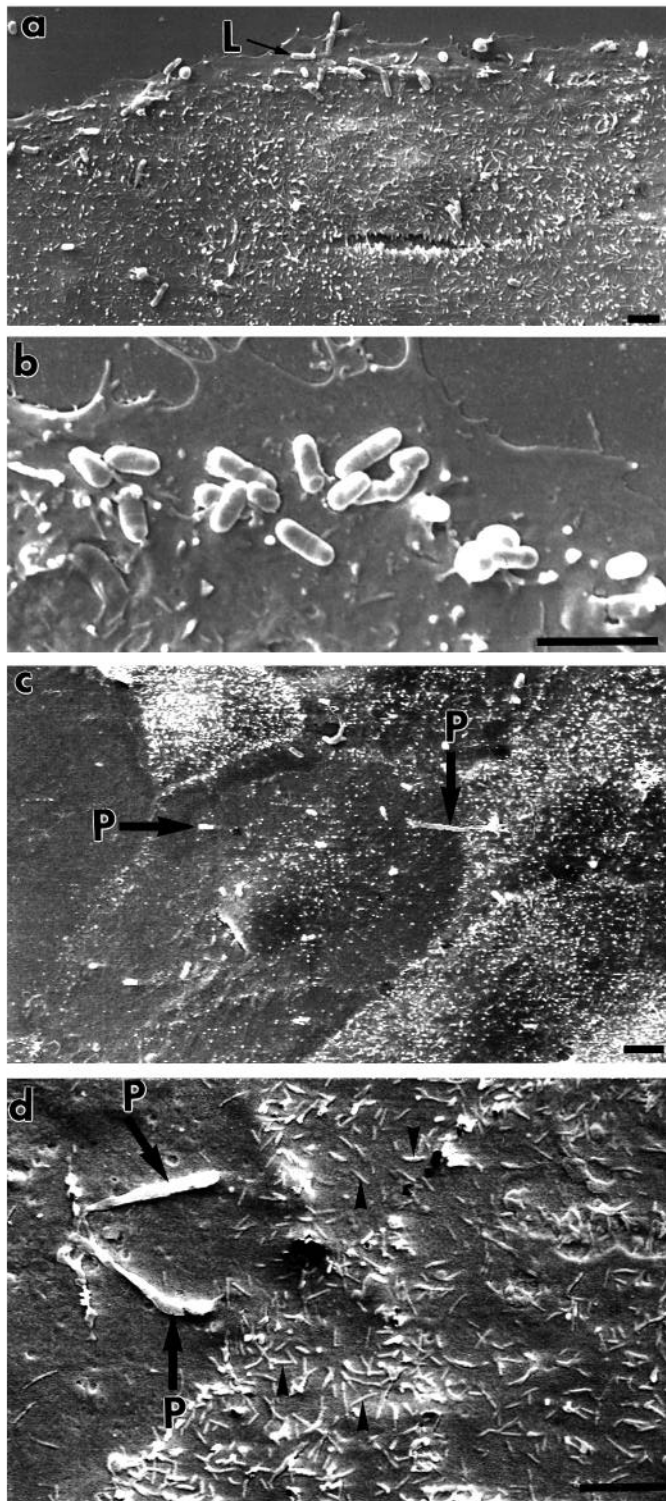
some cells in the more central region of the islets showed long protrusions, but much fewer microvilli than normally found on LLC-PK1 cells. The generation of long cellular projections or protrusions by intracellular *Listeria* has been described as a vehicle for cell-to-cell spreading of this parasite (Tilney and Portnoy, 1989). Thus, these protrusions, as seen by SEM on the cell surface, suggest recent infection from neighbouring cells without involvement of the extracellular environment. Interestingly, we observed that these protrusions were apparently not compatible with a dense population of microvilli on the same cell, indicating that LLC-PK1 microvilli may have to be sacrificed during colonization of an epithelial sheet by vectorial spreading. When inspecting infected islets such as those shown in Fig. 4 d by SEM at lower magnification, we actually observed many groups of cells within these islets with a sparser complement of microvilli but with the typical bacterially induced protrusions (Fig. 7). Such groups of microvilli-depleted LLC-PK1 cells were not detectable in uninfected islets.

DISCUSSION

An especially critical step in *Listeria* pathogenesis is the penetration of the intestinal epithelial surface. The initial site of bacterial entry into the host is still an enigma. In particular, it is not clear whether *Listeria* directly invade the brush border epithelium via the apical microvillar surface that faces the intestinal lumen. One possibility might be that the main route of invasion does not primarily lead through the apical surface of epithelial cells, but involves disruption of the tight junctions, thereby opening paracellular pathways. In this way, the bacteria might gain access to the basolateral surface of the epithelial layer and laterally invade the intestinal epithelium. Alternatively, a preferred route of infection could include highly specialized cells of the intestinal epithelium, such as the M cells of the Peyer's patches, which separate these lymphoid follicles from the intestinal lumen (Bye et al., 1984) and are thought to be involved in the delivery of pathogenic material to the mucosal lymphoid system (Wassef et al., 1989).

Our results using LLC-PK1 islets as a culture model for an intact epithelium show that these cells can be invaded directly, although not primarily through the apical surface. Instead, the bacteria enter preferentially from the periphery of such sheets, where cellular areas devoid of microvilli are exposed. These regions are probably equivalent to the basolateral surface of epithelia in situ. As demonstrated in this analysis, the subsequent colonization of all members of LLC-PK1 epithelial sheets results solely from the vectorial spreading mechanism employed by *L. monocytogenes,* without any further participation of the extracellular milieu. Similar observations have recently been described for the colonization of the human

colonic cell line Caco-2 by *S. flexneri* (Mounier et al., 1992; Vasselon et al., 1992). In both cases the mode of cellular invasion differs significantly from that recently described for *Salmonella*, which can invade polarized cultured epithelial cells from the apical surface by disrupting the microvillus layer (Finlay and Falkow, 1990). In addition, we observed, concomitant with progressing infection of these epithelial sheets by vectorial spreading, a depletion of microvilli from the apical surface of infected cells within these islets. This raises the possibility that localized infections at restricted sites of the host intestinal epithelium could lead to denudation of microvilli from the surface of the infected cells, which might then allow direct invasion by further extracellular bacteria. Such a *Listeria*-induced reduction in microvilli on the surface of intestinal epithelial cells might perturb physiological functions such as nutrient uptake by the brush border epithelium within the gut.



By analysing the composition of microfilamentous structures recruited by *L. monocytogenes* in several epithelial cell lines we have confirmed and extended previous studies on the incorporation of actin-binding proteins into the *Listeria*-induced microfilaments. In addition to the 'ubiquitous' crosslinking protein alpha-actinin, we detected other microfilament-binding proteins in the *Listeria* cytoskeleton, such as fimbrin, villin and ezrin/radixin, that are not general components of all actin filament systems present in animal cells but are restricted to particular subsets. Fimbrin is only found in nonmuscle cells and is confined to cellular projections, like lamellipodia, microspikes, microvilli and stereocilia (see Louvard, 1989). The finding of villin as a component of the *Listeria* microfilament system is particularly interesting, as this protein is synthesized preferentially in specialized epithelial cell types (Robine et al., 1985). However, as *Listeria* also invade non-epithelial cells and exploit the microfilament system of such hosts equally successfully, villin is certainly not essential for the intracellular motility of *Listeria*. Yet, in cells expressing villin, such as LLC-PK1 cells, this protein is readily incorporated into the *Listeria* mobile machinery, which then contains a complement of accessory proteins equivalent to those found in microvilli of the brush border type: fimbrin, villin and ezrin/radixin. This similarity to brush border microvilli is further enhanced by the fact that the actin filaments show the same immunoreactivity as the brush border microvillar core, as demonstrated with the specific chicken antibody against actin. On the basis of these findings it is tempting to speculate that the microfilaments induced by *Listeria* share some structural features with those in brush border-type microvilli. On the other hand, there are obvious differences: brush border microvilli do not contain alpha-actinin, and their core filaments are longer and much more tightly packed than the rather short and more loosely arranged actin filaments that compose the microfilament coat as well as the comet tail of the *Listeria* cytoskeleton (Tilney et al., 1992a).

The *Listeria*-recruited cytoskeletal machinery has two obvious functions: to generate intracellular motility of bacteria

Fig. 6. Scanning electron micrographs of the apical surface of LLC-PK1 cells in *Listeria*-infected islets. (a,b) Thirty minutes after starting the infection, *Listeria* (L) are associated with the surface of peripheral cells; (b) a close up of a peripheral cell, *Listeria* predominantly adhere to the cell surface at the transition between the smooth cell surface and that covered by microvilli. (c) A more central region of an epithelial islet 3 hours after starting the infection. In addition to numerous microvilli, few bacterially induced protrusions (P) are detectable on the surface of one cell. (d) Two protrusions at higher magnification in comparison to the apical microvilli (arrowheads). Note the reduced number of microvilli on the surface of this infected cell in comparison to the neighbouring cell. Bars, 2 μm.

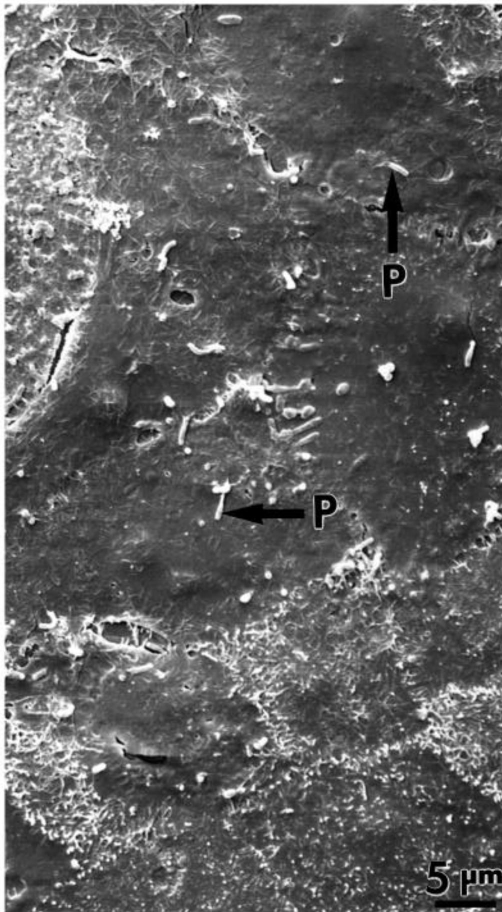


Fig. 7. Scanning electron micrograph of the apical surface of LLC-PK1 cells in a *Listeria*-infected islet 3 hours after starting the infection. In contrast to the cells in the lower part of the micrograph those in the upper part show bacterially induced protrusions (P) and a drastically reduced set of microvilli. Bar, 5 µm.

and, perhaps more importantly, to infect adjacent cells by inducing the uptake of cytoplasmic protrusions harboring motile bacteria. As seen in tissue culture cells, such protrusions can be very long and resemble, in essence, asymmetric projections such as microvilli or pseudopodial extensions. As in these structures, crosslinking of the actin filaments with each other as well as with the plasma membrane apparently stabilizes their microfilament core. Our data on the association of ezrin/radixin, proteins related to band 4.1 (Gould et al., 1989; Wilgenbus et al., 1993), with bacterially recruited microfilaments suggest that these cytoskeletal proteins have a critical function in this latter task. Support for this hypothesis comes from recent transfection experiments (Algrain et al., 1993), which have shown that the amino-terminal portion of ezrin harbours a plasma membrane binding domain, whereas the carboxy-terminal region binds directly to the actin cytoskeleton. In this context, it is noteworthy that we found ezrin/radixin already associated with actin filaments in the bacterial coat and in the comet tail of motile *Listeria* prior to any obvious contact of the *Listeria* cytoskeleton with the plasma membrane. This suggests that ezrin/radixin may actually bind first to actin filaments and subsequently might trigger the interaction of rapidly moving *Listeria* with the inner surface of the plasma membrane, initiating the formation of surface protrusions.

It is now generally accepted that actin polymerization at the interface between the bacterial cell wall and the actin tail provides the propulsive force for the intracellular movement of *L. monocytogenes* (Sanger et al., 1992; Theriot et al., 1992). Recent analysis of the *Listeria* gene products required for intra- and intercellular bacterial movement has shown that: (i) the ActA polypeptide of intracellularly replicating *Listeria* is required for actin accumulation at the bacterial cell surface and thus represents one of the key virulence factors (Domann et al., 1992; Kocks et al., 1992); and (ii) that in infected cells, the ActA protein is restricted to the bacterial surface and not detectable within the comet tail (Kocks et al., 1993; Niebuhr et al., 1993). In addition, the expression of ActA in animal cells in the absence of other bacterial components directs this protein to the mitochondrial membrane, with concomitant actin filament accumulation at this location (Pistor et al., 1994). These results suggest that the ActA protein, once anchored in a membrane, either acts directly as an actin filament nucleator or attracts host cell polypeptides with such a function. The actin subunits needed for this process may be provided by profilactin as a precursor, as suggested by Theriot et al. (1994). By showing that various additional microfilament proteins are also concentrated in the bacterially induced cytoskeleton, we demonstrate that *Listeria* actually require more factors for effective intracellular motility and cell-to-cell spreading.

L. monocytogenes is a very efficient parasite. A large number of different cell types has been found to be susceptible to infection, and the recruitment of the microfilament system seems to be identical in all these cells. Thus, it seems reasonable to assume that the parasite not only uses the host cells' proteins, but also copies its mechanisms for microfilament generation in every detail (i.e. actin nucleation at specific cellular sites, filament growth, packing and crosslinking by associated proteins). Further studies will be necessary to understand these processes, and *Listeria*, by inducing them so effectively, will certainly remain an attractive and very valuable tool in such studies.

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