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NOTES

Involvement of Tubulin and Inhibitory G Proteins in the Interaction of *Listeria monocytogenes* with Mouse Hepatocytes

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Intracellular and cell-to-cell spread of *Listeria monocytogenes* has been considered exclusively actin dependent. By immunocytochemical techniques, we provide evidence for an involvement of inhibitory G proteins and tubulin in "comet tail" formation in *L. monocytogenes*-infected mouse hepatocytes.

Listeria monocytogenes is an opportunistic pathogen responsible for severe food-borne infections in man and animals (7). This microorganism has been extensively used as a model to study host cytoskeleton interactions with intracellular parasites. *L. monocytogenes* enters host cells, where it becomes covered by actin filaments, and moves through the cytoplasm, with unidirectional actin assembly as the driving force, trailing long actin "comet tails" behind (4, 8). Treatment of infected host cells with cytochalasin D affects actin assembly and prevents actin tail and protrusion formation, suggesting that inter- and intracellular spread of *L. monocytogenes* is actin dependent (16). Participation of the microtubular cytoskeleton in intracellular movement of *L. monocytogenes* is controversial. Internalization of *L. monocytogenes* by Caco-2 cells was not inhibited by a microtubule inhibitor, nocodazole, suggesting a microtubule-independent mechanism of phagocytosis (10). In contrast, pretreatment of murine dendritic cells with nocodazole caused marked reduction of bacterial uptake, implying microtubular participation (5). Involvement of both components of the cytoskeleton (microfilaments and microtubules) has been reported for phagocytosis of various bacterial pathogens (2, 11). It is plausible that in phagocytosis, microtubular and microfilamental cytoskeletons act in a cooperative mode.

Soon after systemic infection, listeriosis is manifested primarily in the liver, where the vast majority of bacteria are entrapped (9). We, therefore, attempted to characterize cytoskeletal components involved in *L. monocytogenes* uptake by hepatocytes and to define the composition of the comet tail associated with intracellular and cell-to-cell spread. Parallel localization of the α subunit of inhibitory G proteins ($G_{i\alpha}$) was undertaken, since it participates in signal transduction leading to bacterial uptake and concomitant microtubule rearrangement (for reviews, see references 3 and 6).

L. monocytogenes EGD was grown in tryptic soy broth (Difco) at 37°C for 18 h, and aliquots were frozen at −70°C until use. Mouse hepatocyte line ATCC TIB 75 cells were propagated in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum without antibiotics, as described elsewhere (15). Cells were harvested, seeded in wells of Lab-tec Permanox eight-chamber microscope slides (Nunc, Roskilde, Denmark) at 5×10^4 cells/well in Dulbecco's modified Eagle medium containing fetal calf serum, and incubated for 18 h. Cell monolayers were infected with *L. monocytogenes* for 2, 5, 7, and 24 h at 37°C at an infection rate of 10:1. Thereafter, samples were washed with phosphate-buffered saline (PBS) at 37°C, fixed at room temperature with 2% formaldehyde (freshly prepared from paraformaldehyde in PBS containing 0.5% Tween 20) for 45 min, washed with PBS, and left in PBS at 4°C overnight. For immunolabelling, monolayers were treated as described elsewhere (14). In brief, cells were incubated with primary rabbit polyclonal antibodies (Abs) to tubulin (1:200; Sigma, Deisenhofen, Germany), actin (1:100, Sigma), and α subunits of G_s (1:200) and $G_{i1,2}$ proteins (1:200). Rabbit polyclonal Ab, which recognizes $\alpha_{i1,2}$ subunits of G proteins, and the corresponding control peptide (KNNLKD CGLF) were generous gifts from Peter Gierschik, University of Ulm, or were purchased from Gramsch GmbH (Schwabhausen, Germany). Abs to $G_{s\alpha}$ were purchased from DuPont (Bad Homburg, Germany) and Gramsch GmbH. Secondary Ab was goat anti-rabbit immunoglobulin G (IgG) labelled with peroxidase (Dianova, Hamburg, Germany). The monolayers were viewed and photographed with a Zeiss Axiophot microscope.

The first bacteria within rare, infected hepatocytes were seen as early as 1 to 2 h after infection, but the infection intensity optimal for visualizing the typical comet tails behind bacteria spreading inside and between hepatocytes was not achieved before 5 to 7 h (Fig. 1A). After 24 h of infection, most of the host cells appeared completely destroyed. Only rare, whole cells with actively spreading bacteria were detected (Fig. 1B). Consistent with the findings of published reports (4, 8, 16), *L. monocytogenes* organisms became heavily covered with actin, which formed long tails behind the bacteria (Fig. 1A). Furthermore, in the inner part of this actin tail, two additional components, tubulin (Fig. 1B to D) and $G_{i\alpha}$ (Fig. 1E), were clearly displayed. Phase-contrast microscopy allowed still better visualization of peroxidase-diaminobenzidine immunolabelling of these comet tail components (compare Fig. 1C and D).

Subjected to the same immunostaining procedure described above, *L. monocytogenes* in cytospin preparations did not immunoreact with Abs to actin, $G_{s\alpha}$, and $G_{i\alpha}$ whereas it positively immunoreacted with Abs to tubulin (Fig. 1F). Indeed, tubulin precursors in prokaryotes have been described previously, where they seem to be involved in cytoskeletal functions, in-

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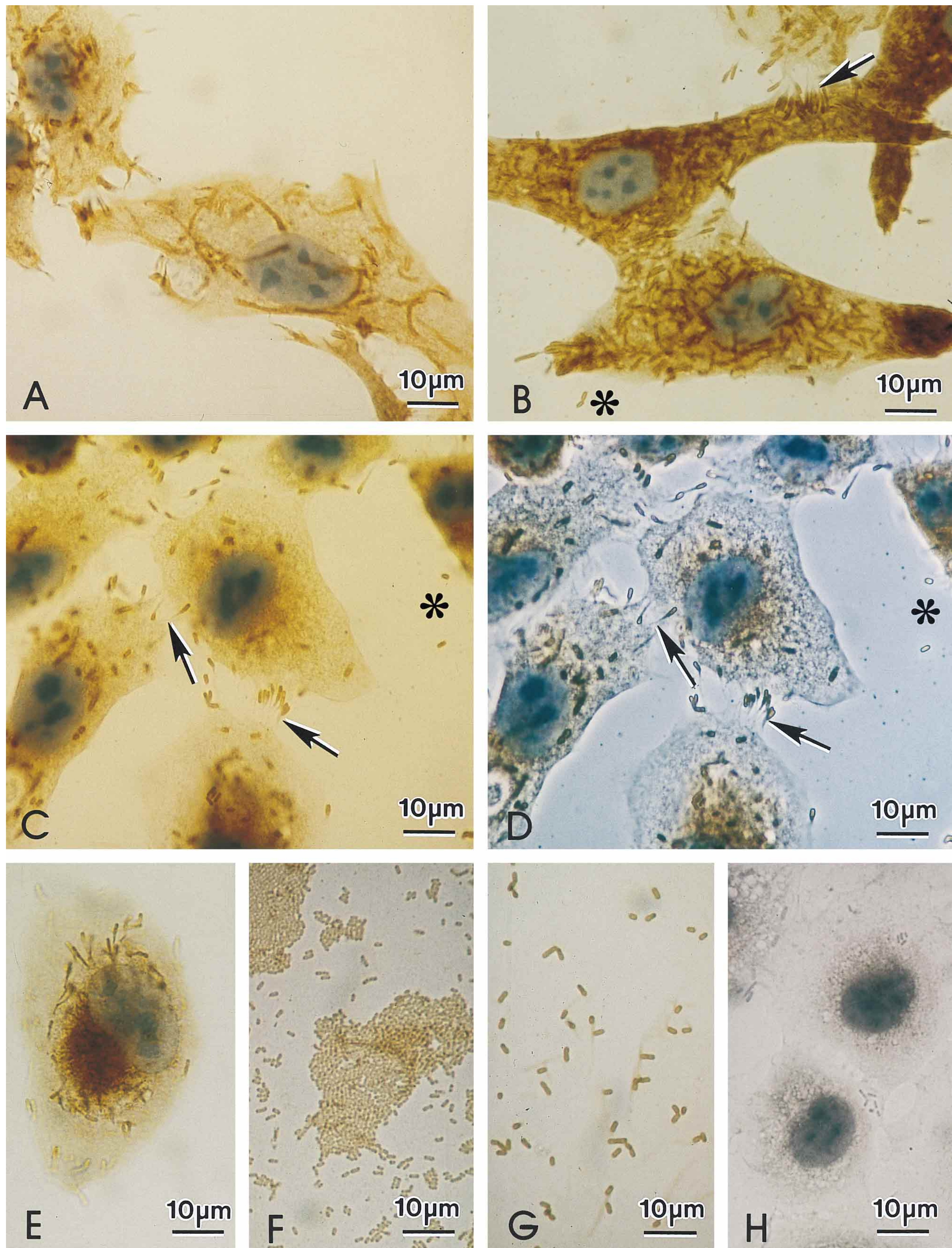

FIG. 1. Immunocytochemical staining of *L. monocytogenes*-infected mouse hepatocytes after 7 h (A, C, D, E, and H) and 24 h (B and G) of infection. (A) Immunolabelling with anti-actin Ab; (B and C) immunolabelling with anti-tubulin Ab; (D) same as panel C but with phase contrast; (E) immunolabelling with anti-$G_{i\alpha}$ Ab; (F) isolated bacteria in a cytospin preparation immunolabelled with anti-tubulin Ab; (G) free bacteria found among lysed cells at a later stage (24 h) of infection immunolabelled with anti-tubulin Ab; (H) control incubation with replacement of primary Ab by rabbit IgG. Arrows point to intercellularly spreading bacteria. Asterisks mark free bacteria between hepatocytes.

cluding intracellular motility or transport activities comparable to those performed by eukaryotic microtubules (reference 1 and citations therein). The polyclonal anti-tubulin Ab used in this study apparently recognizes similar epitopes in bacterial molecules. However, immunolabelling patterns of isolated bacteria in cytospin preparations (Fig. 1F) and of free bacteria found abundantly among lysed cells at a later stage (24 h) of infection (Fig. 1G) were principally distinct from those of the tail-like envelopes surrounding actively spreading bacteria immunostained for tubulin or $G_{i\alpha}$ (Fig. 1B to E).

Results of immunolabelling were similar for both types of Abs to $G_{i\alpha}$, obtained from different sources. Colocalization of tubulin and $G_{i\alpha}$ in the inner zone of the comet tail enveloping *L. monocytogenes* in hepatocytes is consistent with previously reported results of copurification of $G_{i\alpha}$ with microtubules (13). In contrast to $G_{i\alpha}$, $G_{s\alpha}$ was not detected in the comet tail of *L. monocytogenes*, suggesting preferential involvement of $G_{i\alpha}$ in the phagocytosis of *L. monocytogenes*. In controls, where primary Ab was replaced by rabbit IgG (Dianova), no specific immunolabelling was observed (Fig. 1H). Preincubation of Ab to $G_{i\alpha}$ with a 10-fold molar excess of control peptide at room temperature for 2 h decreased immunostaining of $G_{i\alpha}$ (data not shown).

Here, we provide evidence that tubulin is a constituent of the comet tail of *L. monocytogenes* spreading in and between infected hepatocytes. This suggests involvement of microtubules in the intra- and intercellular spread of these bacteria in hepatocytes, as in murine dendritic cells (5). In contrast, Mounier et al. (10) have reported microtubule-independent phagocytosis of *L. monocytogenes* by Caco-2 cells. Pathways for bacterial uptake involving microtubules or microfilaments of the host have been shown to be both cell line and pathogen specific (11). Therefore, it is possible that *L. monocytogenes* employs different strategies to rearrange the cytoskeleton, perhaps depending on the presence and abundance of appropriate receptors in different host cells. It is also possible that nocodazole, used as a microtubule inhibitor, does not inhibit microtubule polymerization equally in all cell types (17). Because the rabbit anti-tubulin Ab immunoreacts with *L. monocytogenes* EGD, we cannot formally rule out the possibility that the bacterial tubulin-like material migrates into the comet tail during its formation. Consequently, the tubulin constituent of the comet tail could be of bacterial and eukaryotic origins.

In conclusion, immunocytochemical labelling of $G_{i\alpha}$ and tubulin suggests that the comet tail of *L. monocytogenes*—at least in hepatocytes—has a more complex composition, encompassing not only actin but also at least two additional components, $G_{i\alpha}$ and tubulin. This implies involvement of tubulin and G_i proteins in the intra- and intercellular spread of *L. monocytogenes* in the liver.

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REFERENCES

1. **Bermudes, D., G. Hinkle, and L. Margulis.** 1994. Do prokaryotes contain microtubules? Microbiol. Rev. **58:**387–400.
2. **Bermudez, L. E., and J. Goodman.** 1996. *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. Infect. Immun. **64:**1400–1406.
3. **Desjardins, M.** 1995. Biogenesis of phagolysosomes: the "kiss and run" hypothesis. Trends Cell Biol. **5:**183–186.
4. **Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wachter, M. Wuenscher, and T. Chakraborty.** 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. **11:**1981–1990.
5. **Guzman, C. A., M. Rohde, T. Chakraborty, E. Domann, M. Hudel, J. Wehland, and K. Timmis.** 1995. Interaction of *Listeria monocytogenes* with mouse dendritic cells. Infect. Immun. **63:**3665–3673.
6. **Hamilton, A. T., Y. Ohmori, J. M. Tebo, S. Narumi, and C. S. Tannenbaum.** 1994. Transmembrane and intracellular signaling events in lipopolysaccharide-stimulated macrophages, p. 83–97. *In* B. S. Zwilling and T. K. Eisenstein (ed.), Macrophage-pathogen interactions. Marcel Dekker, Inc. New York, N.Y.
7. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria, p. 1251–1286. *In* W. E. Paul (ed.), Fundamental immunology. Raven Press, New York, N.Y.
8. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. Cell **68:**521–531.
9. **Mackaness, G. P.** 1962. Cellular resistance to infection. J. Exp. Med. **116:** 381–406.
10. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58:**1048–1058.
11. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko.** 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. USA **90:**6884–6888.
12. **Parton, R. G.** 1991. Endocytosis in polarized cells. Semin. Cell Biol. **2:**387–395.
13. **Roychowdhury, S., and M. M. Rasenick.** 1994. Tubulin-G protein association stabilizes GTP binding and activates GTPase: cytoskeletal participation in neuronal signal transduction. Biochemistry **33:**9800–9805.
14. **Schulze, W., I. B. Buchwalow, W.-P. Wolf, and L. Will-Shahab.** 1994. Comparative immunocytochemical localization of G proteins in the rat heart tissue. Acta Histochem. **96:**87–95.
15. **Szalay, G., J. Hess, and S. H. E. Kaufmann.** 1995. Restricted replication of *Listeria monocytogenes* in a gamma interferon-activated murine hepatocyte line. Infect. Immun. **63:**3187–3195.
16. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite *Listeria monocytogenes*. J. Cell Biol. **109:**1597–1608.
17. **Unger, E., H. Hädrich, S. Rubino, P. Cappucinelly, P. Mühlig, and E. Jelke.** 1982. The microtubule system and the reduplication of microtubule organizing centres in *Dictyostelium discoideum*. Acta Histochem. **XLI:**19–28.

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