Yogi Berra, Forrest Gump, and the discovery of *Listeria* actin comet tails

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ABSTRACT **In 1988, eminent cell biologist Lew Tilney and newly appointed Assistant Professor of Microbiology Dan Portnoy met at a picnic and initiated a collaboration that led to a groundbreaking paper published in *Journal of Cell Biology* entitled "Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*." The paper has been cited more than 800 times, the most of any publication in the careers of both investigators. Using an electron microscope from the Sputnik era, they assembled a stunning collection of micrographs that illustrated how *L. monocytogenes* enters the host cell and exploits a host system of actin-based motility to move within cells and into neighboring cells without leaving the host cell cytosol. This research captured the imagination of cell biologists and microbiologists alike and led to novel insights into cytoskeletal dynamics. Here, Portnoy provides a retrospective that shares text from the original submission that was deleted at the time of publication, along with reviewers' comments ranging from "It is really just a show and tell paper and doesn't have any meat" to "the finding will have major impact in cell biology and in medicine. Potentially, the paper will be a classic."**

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In 1988, I arrived at the University of Pennsylvania as Assistant Professor of Microbiology. My primary research focus was on intracellular pathogens, which then, as now, are responsible for an enormous amount of morbidity and mortality worldwide. The research began during my final year of postdoctoral training at the Rockefeller University and during two subsequent years as an Instructor at Washington University. In St. Louis, we developed quantitative assays to examine the interaction of bacteria and cultured cells, which years later would be referred to as the "bread and butter" of the Portnoy lab. Although *Listeria monocytogenes* was obscure to most cell biologists, and frankly scared many of them, it had been extensively studied for 25 years in a murine model of cell-mediated immunity (Unanue, 1997) and is an important food-borne pathogen (Farber and Peterkin, 1991). However, in 1986, virtually nothing was known about its determinants of pathogenesis or the cell biology of infection, and there was no genetic system to speak of. The first goal was to sort out the nuts and bolts of *L. monocytogenes* pathogenesis, then merge this information with immunological studies and ultimately apply this knowledge to generate vaccines that would be protective against intracellular pathogens.

Prior to my move to the University of Pennsylvania, we did know a few things. We knew that *L. monocytogenes* replicated (doubling time of ~40 min) as rapidly in mammalian cells as in rich bacterial broth and grew in most, if not all, adherent mammalian cells. We also knew that a secreted pore-forming hemolysin called listeriolysin O (LLO) was required for *L. monocytogenes* intracellular growth, and there was evidence that its role was to allow internalized bacteria to escape from a phagosome into the host cell cytosol (Gaillard *et al.*, 1987; Portnoy *et al.*, 1988). By simply observing stained cells infected with *L. monocytogenes*, it was obvious that the bacteria spread directly from cell to cell, even in the presence of gentamicin at levels that killed extracellular bacteria. Remarkably, a single cell could be infected, and by 8 h, 10 cells were infected. The first paper to demonstrate cell-to-cell spread was published in 1986 by Ed Havell while he was studying the interferon response to infection (Havell, 1986). In addition, Chihiro Sasakawa at the University of Tokyo had identified a locus in *Shigella flexneri* essential for cell-to-cell spread (Makino *et al.*, 1986).

One of the first ideas to provide a possible explanation for cell-to-cell spread was suggested to me by Joel Swanson, then Assistant Professor at Harvard and a long-time friend and colleague whose lab was next door when we were postdoctoral fellows at Rockefeller University. Joel posited that spreading might require microtubules and recommended that I examine the effects of nocodozole. Although nocodozole caused the cells to round up, the bacteria still

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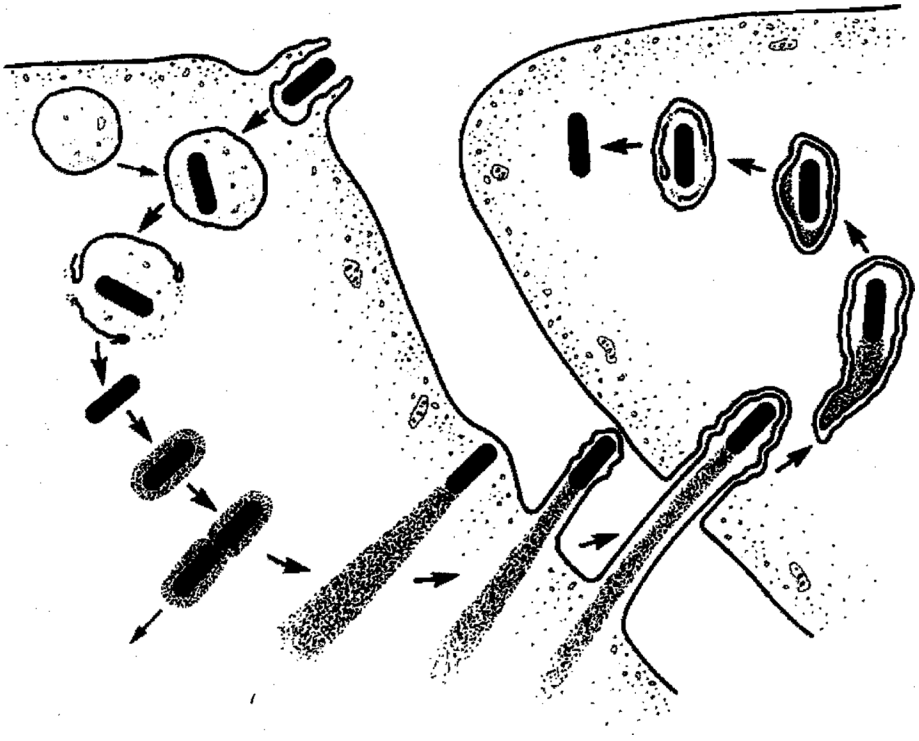

FIGURE 1: Stages in the entry, growth, movement, and spread of *Listeria* from one macrophage to another. Photographs illustrating all these intermediate stages have been presented in the figures in Tilney and Portnoy (1989). With copyright agreement from Rockefeller University Press.

spread cell to cell. Larry Hale, who worked on *S. flexneri* at Walter Reed Army Institute of Research, offered the first substantial clue that led to the discovery by Tilney and Portnoy. Larry told me that spreading of *S. flexneri* was blocked by cytochalasin D, a chemical inhibitor of actin polymerization (Pal *et al.*, 1989). Sure enough, cytochalasin D, at remarkably low concentrations, completely blocked the capacity of *L. monocytogenes* to spread within an infected cell; the bacteria grew as cytosolic microcolonies. Next, I heard through the grapevine that Philippe Sansonetti from the Pasteur Institute presented evidence at a Gordon Conference that intracellular *S. flexneri* were coated in filamentous actin, whereas mutants defective in cell-to-cell spread did not (Bernardini *et al.*, 1989). Finally, before I departed from Washington University, I did a few experiments with John Heuser, a brilliant and eclectic electron microscopist, that provided evidence that *L. monocytogenes* enters the cytosol and becomes enshrouded in host material that we suspected contained actin filaments. Apparently, electron microscopists all seem to know each other, and John told me to look up Lew Tilney when I got to Penn.

As I rarely passed up a party, it was not surprising that I ran into Lew Tilney at a Biology Department picnic on the Penn campus in September 1988. Honestly, as a bacteriologist, I had never heard of him or his classic work on the actin-based acrosomal reaction of starfish sperm (Tilney *et al.*, 1973). I was soon to appreciate that Lew was a monumental figure among cell biologists and a truly colorful and unconventional character. Mentioning that I knew John Heuser, along with having a couple of cold ones, helped break the ice. I shared with Lew all we knew about *Listeria* and the possible role of actin. When I told him about the cytochalasin D results, he grumbled, "People who use inhibitors are inhibited." Nevertheless, he agreed to meet on Monday. Lew later recalled, "Portnoy crashed a department picnic and insisted I look at his damn *Listeria*—I couldn't even spell *Listeria*—then I took one look, and bam, you're hooked" (Powell, 2005).

On Monday morning, I met Lew in his lab. (If he had an office, I never saw it.)

That evening, I laid down some J774 macrophage-like cells onto bacteriologic Petri dishes so that the cells could be easily dislodged, and on Tuesday, we did our first experiment using wild-type and LLO-deficient *Listeria*. I infected and fixed cells in my lab and walked the dishes to Lew's lab, where his long-time technician Pat Connelly did all of the postfixation processing and microscopy, using an electron microscope from the 1950s. We met nine days later to review the results. I had never seen such beautiful micrographs! Lew identified what he later called an actin cloud around the bacteria, which he was sure consisted of actin filaments. He noted, "Of considerable interest is that most of the *Listeria* that are found free in the cytoplasm have now acquired a cloud or mat of material that surrounds them. Higher resolution of this 'mat' shows that it is fibrillar in nature, being composed of dots (the filaments cut in transverse section) and short segments of filaments (oblique section). These filaments tightly surround the free *Listeria*" (Tilney and Portnoy, 1989).

However, we were unable to determine the spatial relationship of the actin filaments to the bacteria since we had scraped the infected cells from the dish. Lew decided that we should do a time course and fix in situ on plastic tissue culture-treated Petri dishes. Again, I laid down cells on Monday and brought him the fixed samples on Tuesday. Most of the figures in Tilney and Portnoy were derived from this experiment, and here he coined the term "comet tails." In subsequent experiments, we verified that the filaments were actin and examined their polarity by staining with the S1 fragment of myosin. The results excited me, but I did not appreciate the big picture until Lew sent me a draft of a manuscript from Woods Hole during winter break. Of importance, he had an artist draw a cartoon derived from the micrographs (Figure 1). This figure eventually landed in many textbooks of microbiology and cell biology and seems to be used during the introduction to almost every *Listeria* seminar. The impact of this figure cannot be overestimated.

Fortunately, I saved a folder that contains the original drafts of the paper, reviews, and rebuttals. The first draft blew me away. The writing was masterful although highly unconventional. One line in the *Results* section, which made it to the published version, reads, "Thus, this insidious beast has managed to multiply and spread cell-to-cell without ever leaving the cytoplasm of its host." However, the phrase, "Machiavellian deviousness," which Lew wrote to describe intracellular parasites in a draft of the *Discussion* section, had to go. We submitted the paper to *Cell* in February, and it was returned in March, rejected. Here is one of the reviewer's comments: "The paper is technically flawless and of good quality, albeit a bit lengthy. However it just is a 'show and tell' paper and really doesn't have any meat. It is not the type of paper readers would expect to see in *Cell*. The system has the potential for some very exciting cell biology of the sort Tilney lists in the last paragraph of his discussion, but it hasn't happened yet. My suggestion would be to reject this one but to encourage Tilney to submit the follow-up paper (and perhaps

hint that it might be treated favorably) because it can be expected to be even more exciting."

Another reviewer wondered why we had not tried one of the "modern fixatives that preserve actin filaments better." However, our study used Lew's magical cocktail consisting of glutaraldehyde and osmium tetroxide (called "the mixed fix"). This ideal brew allowed most of the cytosolic components to escape fixation while highlighting cellular membranes and actin filaments.

After a failed attempt at a rebuttal to *Cell*, we sent the manuscript to the *Journal of Cell Biology*. Here, one of the reviewers also believed the paper should be rejected, because it was "really a look and see report." This reviewer recommended, "For publication in the *JCB* I would expect some more experimentation on some aspect of the cell biology of the system." Fortunately, the other reviewer saved the day: "The observations constitute a really new finding—nobody knew this before! What's more, the finding will have a major impact in cell biology and in medicine. Potentially, the paper will be a classic." The second reviewer had a few memorable comments about the other reviewers; one of my favorites: "What a disaster that many good scientists no longer recognize the validity and clarity of information that we obtain with our sensory organs—how do these scientists manage to get around on a day-to-day basis?" Fortunately, the editor, Tom Pollard, accepted the paper. One note: Lew often included Pat Connelly on papers, but in this case, he did not. He told me that Tilney and Portnoy would benefit my career more than Tilney *et al*. Thank you, Lew!

The final paragraph of the original discussion, written entirely by Lew, was ultimately deleted by the editors, but provides such insight that I include it here verbatim:

> As with most scientific studies we are left with more questions than we started with. For example, if the membrane of a phagolysosome is dissolved by the hemolysin of the bacterium, why is the host cell not sicker because of the release of the lysosomal enzymes into the cytoplasm? In the same vein, since hemolysin of *Listeria* has an pH optimum of 5.5 with no detectable activity at 7.0 (Geoffroy *et al.*, 1987), how can *Listeria* get out of the double membrane compartment it is in when it spreads from one cell to the next? The hemolysis should be in the inner vacuolar (formerly pseudopod plasma membrane of the old host cell) membrane, but lysosomes would only fuse and acidify the outer portion of the phagosome. How does the new host macrophage 'know' to phagocytose only the specific pseudopods of donor macrophages that have a *Listeria* at their tips? How does it break off from the old host? What is the time course of the events of *Listeria* spreading, seconds, minutes, hours? Does the cell wall of *Listeria* bind to actin filaments or does it induce polymerization of monomeric actin to filaments and if so, how? How does a comet tail form from a circular mat of actin filaments around a *Listeria*? How does the comet plus tail move to the cell surface and make a pseudopod? Why is the tail of the comet composed of randomly oriented filaments rather than a bundle of cross-bridged filaments and why does the presentation of the *Listeria* to a new host occur at the tip of a pseudopod rather than the tip of a microvillus, a structure one would imagine would be easier to build? Many of these questions can be answered by looking at living cells (Schaechter *et al.*, 1957) and will give us information not only on *Listeria* and its proliferation and for that matter certain intracellular parasites generally, but also help cell biologists learn more about the cell biological processes. We live in fascinating times.
>
> Can you imagine writing his final sentence today?

There are a few reasons this research garnered so much attention. For one, Lew was well known, respected, and had a dedicated fan club. Also, an article in *Nature*'s News and Views entitled "The pushy ways of a parasite" appeared the month following the publication of Tilney and Portnoy and highlighted the work with a reproduction of the model figure from the paper (Donelson and Fulton, 1989). In his rebuttal to *Cell*, Lew had argued with the editor that the paper deserved publication because of all the attention is was generating: "the reviewers have no way of knowing this, [but] our work has already fascinated a large number of biologists because Portnoy loves to give seminars and has stimulated a number of investigators to carry this project forward." Indeed, between 1989 and 1992, I gave 45 seminars, including 20 at national and international meetings. The notoriety was gratifying, but unfortunately, Sansonetti's and Sasakawa's work on *S. flexneri* had not been acknowledged in the publication or the News and Views (Bernardini *et al.*, 1989; Makino *et al.*, 1986).

One of the cell biologists intrigued by our findings was Tim Mitchison, who heard about the work from Lew during the summer at Woods Hole. Tim and Lew debated whether the tails moved, perhaps using an unknown motor protein, or remained stationary in the cell cytosol. Tim, then Assistant Professor at the University of California, San Francisco, shared the work with his doctoral student, Julie Theriot. In October 1990, Julie proposed as part of her doctoral research to use *Listeria* to identify host factors that control actin polymerization to complement her primary research on the control of actin polymerization at the leading edge of keratinocytes. Julie and Tim used fluorescence photoactivation and time-lapsed video microscopy to study the dynamics of actin filament polymerization in living cells (Theriot and Mitchison, 1991). Lew suggested that I visit Julie and Tim, so in November 1991, I flew to San Francisco, bacteria in hand (have *Listeria*, will travel) and initiated what would become a long-term collaboration with Julie that has lasted from her time as a Whitehead Fellow through today, as a faculty member down the road at Stanford. On the first morning, we infected PtK2 cells, and using their home-made video system, Julie filmed what remains among the best movies of *Listeria* actin-based motility (www.youtube.com/watch?v=sF4BeU60yT8). The next day, Julie did the experiment that led to a *Nature* paper, coauthored with Lew, entitled, "The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization" (Theriot *et al.*, 1992). Here she demonstrated that the comet tails do not move and that the actin filaments are rapidly turning over, with a half-life of ~30 s. She effectively argued that actin polymerization alone could provide the propulsive force necessary for actin-based motility. These observations, combined with the work of Fred Southwick and the Sangers, clearly showed that actin filaments polymerized at the interface of a bacterium and its actin tail and that the actin filaments in the tail were stationary (Dabiri *et al.*, 1990; Sanger *et al.*, 1992). Also in 1992, Pascale Cossart at the Pasteur Institute and Trinad Chakraborty, Werner Goebel, and Jurgen Wehland in Germany made the critical codiscovery of the *Listeria* ActA protein (Domann *et al.*, 1992; Kocks *et al.*, 1992). Each group, along with Julie Theriot and Greg Smith in my lab, later used novel approaches to show that ActA is not only necessary, but also sufficient, to mediate actin polymerization (Brundage *et al.*, 1993; Pistor *et al.*, 1994; Friederich *et al.*, 1995; Kocks *et al.*, 1995; Smith *et al.*, 1995).

In a second paper I coauthored with Lew (Tilney et al., 1990), he proposed that *Listeria* might lead us to the elusive actin nucleator: "We have stumbled upon a biological system in which we have an excellent chance of isolating, purifying, and characterizing

a natural actin filament nucleator." A few years later, Matt Welch, then postdoctoral fellow in the Mitchison lab and now a close colleague at University of California, Berkeley, used *Listeria* overexpressing the ActA protein and conventional biochemistry to discover that the Arp2/3 complex was the host cell nucleator (Welch *et al.*, 1997b). Matt later established that purified ActA was a direct activator of the complex and the founding member of what are now called actin nucleation promoting factors (NPFs; Welch *et al.*, 1998). Matt and others showed that ActA is a molecular mimic of cellular NPFs, such as proteins in the WASP family (Skoble *et al.*, 2000; Boujemaa-Paterski *et al.*, 2001). A few years later, Mary-France Carlier at the Centre National de la Recherche Scientifique in France accomplished in vitro reconstitution of *Listeria* actin-based motility using the Arp2/3 complex, actin, cofilin, and capping protein (Loisel *et al.*, 1999).

By 1993, Lew was out of the *Listeria* business, although he continued his career as one of the world's great observationists, working on, among other things, *Drosophila* wing hairs and bristle cells (Tilney and DeRosier, 2005). He retired a few years ago and currently lives a bit reclusively in Massachusetts and chose not to be a coauthor of or comment on this retrospective, but he did thank me for thinking of him. In 1997, I moved to Berkeley and continued to work on the role of ActA until 2003 (Skoble *et al.*, 2000, 2001; Lauer *et al.*, 2001; Auerbuch *et al.*, 2003), then changed to a more immunological research focus (Witte *et al.*, 2012). One particularly gratifying consequence of the actin-based motility research was the observation that ActA-deficient strains make extremely potent and safe immunotherapeutic vaccines (Brockstedt *et al.*, 2004), which have shown promising results in clinical trials for pancreatic and other cancers (Guirnalda *et al.*, 2012; Le *et al.*, 2012).

I suspect that the reader may be wondering about the title of this retrospective. As one interested in pathogenic microorganisms, I view myself as Forrest Gump in the world of cell biology, lucky to have worked and collaborated with such creative and accomplished cell biologists like Lew, Julie, Matt, and Tim. For me, microbial pathogenesis "is like a box of chocolates; you never know what you are gonna get." But most remarkable to me is that so much of what we learned came from simply looking through the microscope. As Yogi Berra famously remarked, "You can observe a lot by watching."

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