Actin Filaments and the Growth, Movement, and Spread of the Intracellular Bacterial Parasite, *Listeria monocytogenes*

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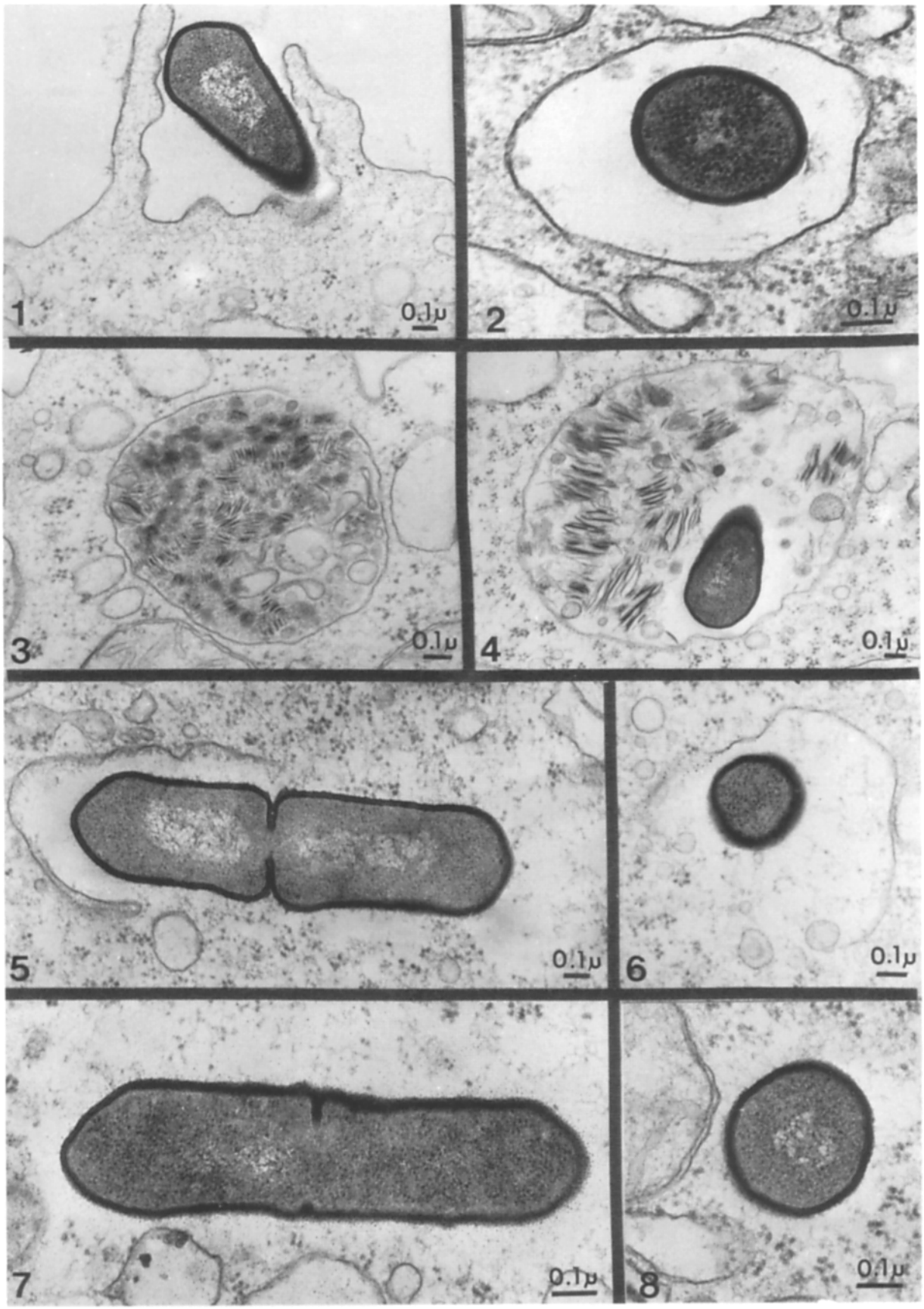
Abstract. *Listeria monocytogenes* was used as a model intracellular parasite to study stages in the entry, growth, movement, and spread of bacteria in a macrophage cell line. The first step in infection is phagocytosis of the *Listeria*, followed by the dissolution of the membrane surrounding the phagosome presumably mediated by hemolysin secreted by *Listeria* as nonhemolytic mutants remain in intact vacuoles. Within 2 h after infection, each now cytoplasmic *Listeria* becomes encapsulated by actin filaments, identified as such by decoration of the actin filaments with subfragment 1 of myosin. These filaments are very short. The *Listeria* grow and divide and the actin filaments rearrange to form a long tail (often 5 μm in length) extending from only one end of the bacterium, a "comet's tail," in which the actin filaments appear randomly oriented. The *Listeria* "comet" moves to the cell surface with its tail oriented towards the cell center and becomes encorporated into a cell extension with the *Listeria* at the tip of the process and its tail trailing into the cytoplasm behind it. This extension contacts a neighboring macrophage that phagocytoses the extension of the first macrophage. Thus, within the cytoplasm of the second macrophage is a *Listeria* with its actin tail surrounded by a membrane that in turn is surrounded by the phagosome membrane of the new host. Both these membranes are then solubilized by the *Listeria* and the cycle is repeated. Thus, once inside a host cell, the infecting *Listeria* and their progeny can spread from cell to cell by remaining intracellular and thus bypass the humoral immune system of the organism. To establish if actin filaments are essential for the spread of *Listeria* from cell to cell, we treated infected macrophages with cytochalasin D. The *Listeria* not only failed to spread, but most were found deep within the cytoplasm, rather than near the periphery of the cell. Thin sections revealed that the net of actin filaments is not formed nor is a "comet" tail produced.

THERE is an enormous biological range of organisms that function as intracellular parasites from protozoa to tiny RNA viruses. Some of these organisms are quite intimidating and cause serious human illnesses; they include viruses (rabies, yellow fever, smallpox, influenza, AIDS, to name a few), bacteria (acute diarrhea, *Shigella flexneri*; scrub typhus, *Rickettsiae tsutsugamushi*; Rocky Mountain spotted fever, *Rickettsiae rickettsiae*; tuberculosis, *Mycobacterium tuberculosis*; leprosy, *Mycobacterium leprae*; Legionnaires disease, *Legionella pneumophila*), and protozoa (Chagas disease, *Trypanosoma cruzi*; Kala azar, *Leishmania donovani*; and malaria, *Plasmodium falciparum*). Other parasites are much less dangerous and in fact can benefit the host; e.g., the symbiotic relationship between *Chlorella* (a green algae) and an epidermal cell of the freshwater hydra, *Hydra viridis*. The study of intracellular parasites illustrates the fascinating diversity of mechanisms that have evolved to allow this interaction to occur and accordingly helps us to learn more about the physiology of the host cells. Furthermore, by discovering how the parasite evades the immune system of the host, we may be able to devise suitable therapeutic protocols to control infection and/or proliferation of life threatening parasites.

It is useful at the outset to separate intracellular parasites into two groups (Moulder, 1985). One group is comprised of parasites which throughout their lives are enclosed by a membrane of the host, an endosome; e.g., *Mycobacterium, Legionella, Chlorella,* and *Plasmodium*. The second group consists of parasites that grow, feed, and replicate within the cytoplasm proper, not confined by a vacuolar membrane of the host. These include *T. cruzi, S. flexneri, R. tsutsugamushi,* and *L. monocytogenes*. These are particularly insidious as not only are they protected from the immune system of the host, being intracellular, but, as will be shown below, at least for one organism (*L. monocytogenes*), are also transmitted from cell to cell without ever leaving the host's cytoplasm.

In the past 10 years, there have been two new approaches to the study of how intracellular parasites adapt to the cytoplasm of the host (Edelson, 1982; Moulder, 1985). The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first is the use of macrophage cell lines rather than parts of infected organisms which greatly simplifies investigations into the cell biology of the parasites. The second is the use of transposon mutagenesis and techniques of molecular biology to study infection, multiplication, and spread of mutant parasites in these cell lines.

Listeria monocytogenes is an intracellular parasite that penetrates, multiplies, and is transmitted from cell to cell in macrophage, fibroblast, and enterocyte cell lines. It is a model for understanding other cytoplasmic parasites (Gaillard et al., 1986, 1987; Havell, 1986, Kuhn et al., 1988; Portnoy et al., 1988). The parasite is a pathogen that is transmitted in the human population by contaminated milk products, but is not extremely toxic to humans although it can be serious and cause death in pregnant women and neonates and in individuals whose immune systems are compromised (Linnen et al., 1988) and, very rarely, in apparently healthy individuals. Although it has been studied by a number of investigators over the past 25 years as a model for cell-mediated immunity (Hahn and Kaufman, 1981; Mackaness, 1962), so that there is a wealth of information on the immune response to *Listeria*, the cell biology of infection has been virtually ignored. Some information is available on the entry of *Listeria* into the cytoplasm of a human carcinoma cell line (Gaillard et al., 1987), but nothing is known of its movement across the cell and spread of infection into adjacent cells.

In this report, we will describe the penetration of the bacterium into the cytoplasm, the movement of the replicating bacteria to the cell surface of the host, and the spread of the bacteria into neighboring cells. What we found was unexpected. Following phagocytosis by the host macrophage and escape from the phagocytic vacuole *Listeria* becomes coated with a large population of actin filaments. These filaments rearrange to form a large "tail" of actin up to 5 μm long. This complex then moves to the surface of the host cell and produces a large microvillus or, more accurately, a pseudopodal projection with the *Listeria* now at its tip. This pseudopod touches a neighboring cell that phagocytoses the apical end of it. The bacterium now dissolves the membranes surrounding it (the former pseudopod membrane of the first host and the phagocytic, vacuolar membrane of the new host), presumably by its hemolysin, and the cycle is repeated.

Because the cytoskeleton plays such an important role in the spread of infection, our study should be of interest to two types of investigators. First, it should be important to those concerned with stages in the cell biology of infection by parasites and second it should be exciting to cell biologists who want to know how actin filaments become organized in cells and, more specifically, how actin filament distribution and length is regulated.

Materials and Methods

Bacterial Growth Conditions

The virulent *L. monocytogenes* strain 10403S (Bishop and Hinrichs, 1987) and its isogenic, avirulent, hemolysin minus mutant strain DPL-L215 (Portnoy et al., 1988) were used in this study. Strain 10403S belongs to serotype 1. It is resistant to 1 mg/ml of streptomycin and its LD_{50} for mice is 3×10^4. Bacteria were grown on brain heart infusion agar and broth (BHI; Difco Laboratories Inc., Detroit, MI) at 30°C. Stock cultures were kept as suspensions of cells at −70°C in 50% glycerol.

Tissue Culture Cells and Growth Medium

The macrophage-like cell line J774 (Ralph et al., 1975) obtained from J. Unkeless (Mount Sinai Medical School, New York, NY) were grown in spinner flasks in DME (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS and maintained in the presence of penicillin (100 U/ml) and streptomycin (10 μg/ml).

Infection

L. monocytogenes was grown overnight in BHI broth at 30°C to a density of 2×10^9/ml. 1 ml of culture was sedimented in a microfuge tube (14,000 *g*) for 1 min; the supernatant was discarded; and the pellet was washed once in 1 ml of PBS, pH 7.4. Monolayers of J774 cells were infected with various numbers of bacteria depending upon the duration of the experiment to be performed. Therefore for a 30-min infection, 4×10^7 bacteria were added per 5 ml of culture; for a 2-h infection 2×10^7; and for a 4-h infection 5×10^6. Approximately 10% of these bacteria were internalized by 30 min. After the initial 30-min infection, monolayers were washed three times with 37°C PBS followed by the addition of 5 ml of prewarmed growth medium containing gentamicin sulfate added to a final concentration of 5 μg/ml. This concentration of gentamicin kills all extracellular *Listeria* but does not harm intracellular *Listeria* (Portnoy et al., 1988).

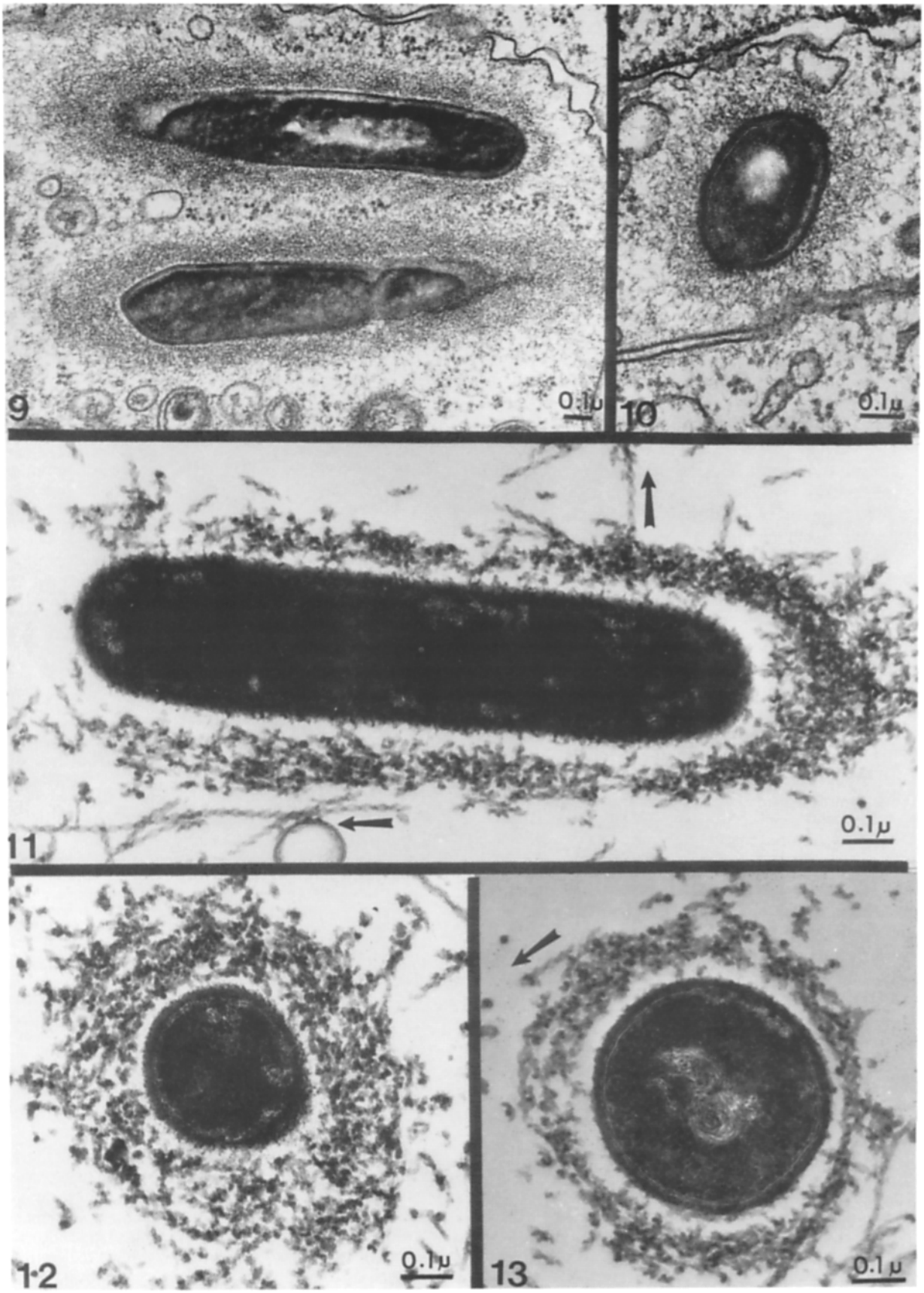
Cytochalasin

Cytochalasin D (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide. Macrophages were infected with *Listeria* for 30 min, washed three times with PBS at 37°C, followed by the addition of prewarmed growth medium containing gentamicin sulfate. 1 h after the beginning of the initial infection cytochalasin D at 0.5 μgm/ml was added. 3 h later, the macrophages, still in the cytochalasin, were fixed and processed for light and EM. Other preparations were left in cytochalasin for 7 h before fixation.

Electron Microscopic Observations

Two types of preparations were examined. In the first, cell monolayers were grown in 60-mm petri dishes (Lab-tek, Baxter Healthware, Inc., Edison, N.J.) containing 5 ml of the appropriate medium without antibiotics. J774 is easily dislodged from these dishes. At various times after infection, macrophages grown in monolayers on 60-mm petri dishes were removed by incubating for 10 min in PBS at 4°C. The macrophages were pelleted at 700 *g* in conical centrifuge tubes and the pellets fixed. For the second procedure, macrophages were grown in plastic tissue culture petri dishes (25 mm^2), infected and at selected times after infection the supernatant was gently discarded and the macrophages fixed in situ. All additional steps in processing were carried out in the petri dishes with the macrophages still attached. Fix-

Figures 1–8. Thin sections through macrophages that were infected with *Listeria* for 30 min. (*1*) *Listeria* on the surface of a macrophage cell. (*2*) A *Listeria* that has been internalized in an endosome. Note a membrane completely encircles it. The bulk of the *Listeria* at this stage are in endosomes like these. (*3*) A member of the lysosome family containing membranous material. (*4*) *Listeria* are often found within these lysosomal bodies, presumably because of fusion of an endosome like that in Fig. 2 with a lysosome as in Fig. 3. (*5*) A *Listeria* that is only partially surrounded by an endosomal (lysosomal) membrane. Note that the membrane is intact on the left but *Listeria* is in direct contact with the cytoplasm on the right. The membrane on the right has disappeared, presumably having been solubilized by the bacterial hemolysin. The cytoplasm that is in direct contact with the *Listeria* is often free of formed organelles such as ribosomes (*far right*). (*6*) Cross section of a *Listeria* that has partially solubilized the endosomal membrane at the base of the figure. (*7*) Longitudinal section through a *Listeria* that is completely free of its endosomal membrane and is free in the cytoplasm. (*8*) Cross section of a *Listeria* that is free in the cytoplasm. Bars, 0.1 μm.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ation for both preparations was carried out by the addition of a freshly prepared solution containing 1% glutaraldehyde (from an 8% stock supplied by Electron Microscopy Sciences, Fort Washington, PA), 1% OsO_4 and 0.05 M phosphate buffer at pH 6.3. Fixation was carried out on ice (4°C) for 40 min. The preparation was then washed three times with water (4°C) to remove excess phosphate and then en bloc stained in 0.5% uranyl acetate overnight. The specimens were then dehydrated in acetone or alcohol (for those still attached to the petri plates) and embedded in epon. Thin sections were cut with a diamond knife, picked up on uncoated grids, and stained with uranyl acetate and lead citrate. The sections were examined with an electron microscope (200; Philips Electronic Instruments, Inc., Mahwah, NJ).

For decoration with subfragment 1 of myosin (S1),[1] the petri plates were incubated in 1% Triton X-100 in 30 mM Tris and 3 mM $MgCl_2$, pH 8.0, for 10 min at 4°C to remove the membranes. The cytoskeletons were then incubated in a solution containing 5–8 mg/ml S1 in 0.1 M phosphate buffer at pH 6.8 for 30 min at 4°C, then washed in 0.1 M phosphate buffer to remove the unbound S1 and fixed in a solution containing 1% glutaraldehyde, 2% tannic acid, and 0.05 M phosphate buffer at pH 6.8 for 30 min. The preparation was then washed in 0.1 M phosphate buffer and postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 6.3, for 30 min at 4°C, rinsed in water, and dehydrated and embedded as mentioned above. The S1 was prepared from rabbit skeletal muscle and quick frozen in small aliquots at high concentration, 70 mg/ml, in liquid nitrogen. These aliquots were stored in liquid nitrogen and thawed just before use.

Results

30 Min after Infection

By 30 min, most of the bacteria encountered in our thin sections lie within the cytoplasm of the macrophage encapsulated by a membrane, although a few are seen free in the cytoplasm proper, not surrounded by a membrane (Figs. 2–8). We also find an occasional bacterium on the surface of the macrophage closely associated with (bound to) the plasma membrane (Fig. 1). From earlier studies on *Listeria* and other bacteria (Gaillard et al., 1987; Portnoy et al., 1988; Racz et al., 1972; Sansonetti et al., 1986; Takeuchi, 1967; Takeuchi et al., 1965), we can readily order the events of entry of the *Listeria* into the cytoplasm of the macrophage host.

The first step seems to be an association of *Listeria* with microvilli on the plasma membrane (Fig. 1). They then become internalized by the cell by phagocytosis and thus reside in a membrane-limited vacuole or endosome (Fig. 2). A common organelle found in this macrophage cell line is a membrane-limited body packed with debris (Fig. 3). These appear to be part of the lysosome family and have been called multivesicular bodies. We often find in a phagosome in which *Listeria* is still residing with what appears to be membranous debris similar to that in the lysosomal bodies just mentioned (Fig. 4). The implication is that there has been a fusion of the lysosome and the phagosome, although the combined "phagolysosomes" are larger than might be expected from such a fusion (cf. Fig. 3 with Fig. 4). The debris in the phagolysosome is less compact and seems to be undergoing dissolution as the vesicles are often broken.

The next step is the escape of the *Listeria* from its membrane encapsulation in the phagosome (or phagolysosome) to become free in the cytoplasm. This occurs by the breakdown or dissolution of the phagosome membrane (Figs. 5 and 6) beginning at one margin and eventually including the whole membrane. Host ribosomes, mitochondria, endoplasmic reticulum, and small vesicles can now approach the cell wall of *Listeria* although in many cases we see a small clear zone around the newly liberated bacterium (Figs. 5, 7, and 8). Examples such as Fig. 7 show that some of the *Listeria* are dividing.

It has recently been reported (Gaillard et al., 1987; Kuhn et al., 1988; Portnoy et al., 1988) that *Listeria* lacking hemolysin fail to grow in tissue culture cells or in vivo. This seems to be because of a failure to dissolve the endosomal membrane as mutants lacking hemolysin do not disrupt the endosome membrane and accordingly do not escape from the endosome into the cytoplasm (data not shown). Similar observations have been made by Gaillard et al. (1987).

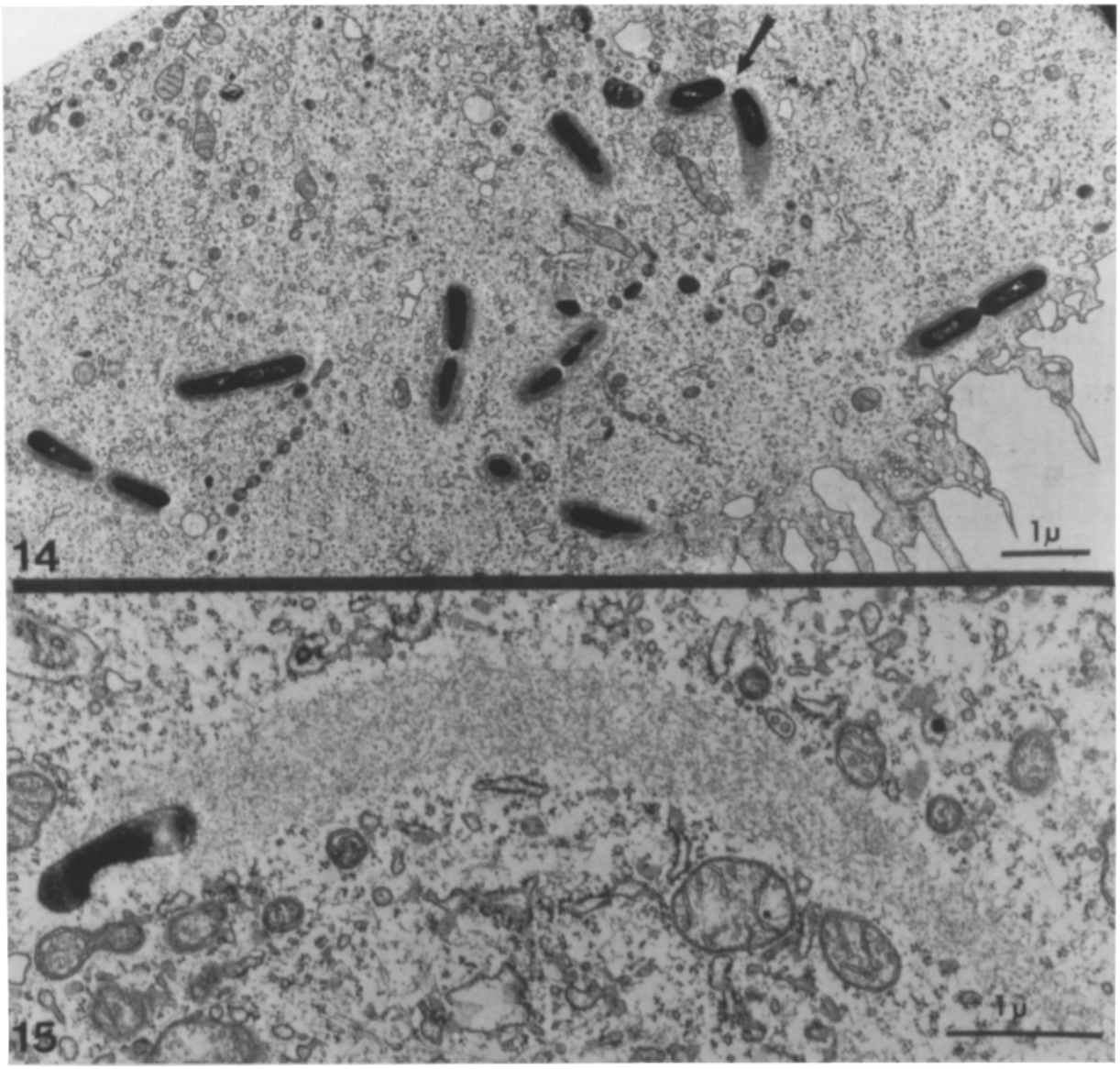
30 Min of Infection Followed by 90 Min of Growth

We infected the macrophages with *Listeria* for 30 min, then washed the macrophages and incubated them in the antibiotic, gentamicin, before fixation 90 min later. Gentamicin kills *Listeria* that have not been internalized (phagocytosed) into the macrophages, but does not affect the *Listeria* that reside within the macrophages (Portnoy et al., 1988). In essence, we are carrying out a "pulse-chase" procedure. Using such a procedure, we now find that the bulk of the *Listeria* are located free in the cytoplasm, not enclosed by a membrane. Thus, in one section through a number of infected cells in which we saw 29 *Listeria*, 31% were still enclosed in an endosome, while the remaining 69% were free in the cytoplasm. 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 (Figs. 9 and 10). 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*.

To achieve higher resolution images of this mat of filaments and to identify them, we first extracted infected macrophages with the detergent, Triton X-100, and "negatively stained" them during fixation with tannic acid. In this case, the mat is composed of fine filaments ~55 Å in diameter (data not shown). We identified these filaments as actin fila-

1. *Abbreviation used in this paper*: S1, subfragment 1 of myosin.

Figures 9–13. Thin sections of portions of macrophages that have been infected with *Listeria* for 30 min, then washed and incubated in media containing gentamicin for 90 min before fixation. (*9*) Nearly longitudinal section through two *Listeria*. Of interest is the cloud of finely filamentous material that encircles each. (*10*) Transverse section of a *Listeria* with its cloud of finely filamentous material. (*11–13*) Macrophages infected with *Listeria* were extracted with the detergent, Triton X-100, then incubated with S1. The fine filaments surrounding the *Listeria* in the previous two figures decorate with S1. They become thicker in transverse section and in longitudinal section they display the arrowhead profile (*arrows*). This identifies them as actin filaments. Of interest is that most of the filaments whether in longitudinal (Fig. 11) or transverse (Figs. 12 and 13) section of the bacteria appear to be cut in transverse section. Bars, 0.1 μm.



Figures 14 and 15. Thin sections of portions of macrophages infected for 4 h with *Listeria*. These macrophages were fixed as monolayers still attached to the dishes. (*14*) At this stage in the infection most of the *Listeria* have just divided. They are all encircled by actin filaments. The pair of *Listeria* indicated by the arrow have started to develop a tail of actin filaments with none at their heads. (*15*) A *Listeria* with a long tail of actin filaments. Bars, 1 μm.

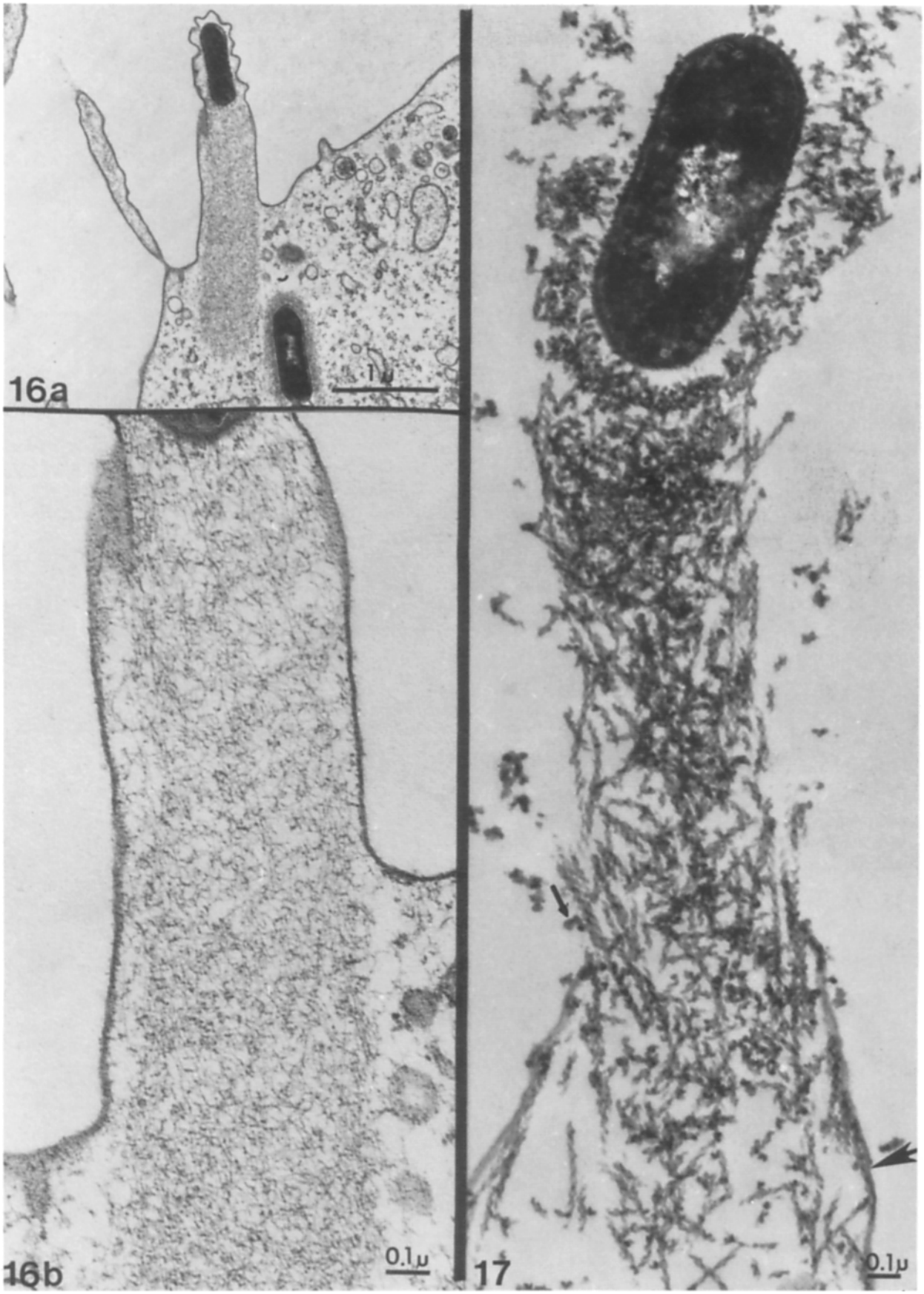
ments by "decorating" them with S1. We find that the filaments decorate and/or increase in diameter (Figs. 11–13). Both features identify the 55-Å filaments as actin filaments. Because the filaments are packed so densely around the *Listeria*, it is often difficult to determine the polarity of the filaments because of superposition of filaments over each other in the same section, but some can be seen (Figs. 11 and 13, *arrows*).

Ultimately, it will be important to determine what controls the distribution of the actin filaments around the *Listeria*. As a first step, it is essential to describe accurately how long these filaments are and how they become located immediately around the *Listeria* as a cloud, not grading out into the rest of the cytoskeleton of the macrophage. In our S1 decorated preparation (Figs. 11–13), both in longitudinal and transverse sections through the bacteria the overwhelming majority of the actin filaments are cut in transverse sections. The only possibilities are that either the actin filaments are very flexible, or they are very short. The latter is most likely the case as actin is known in other systems to be rigid and is sheared readily in vitro.

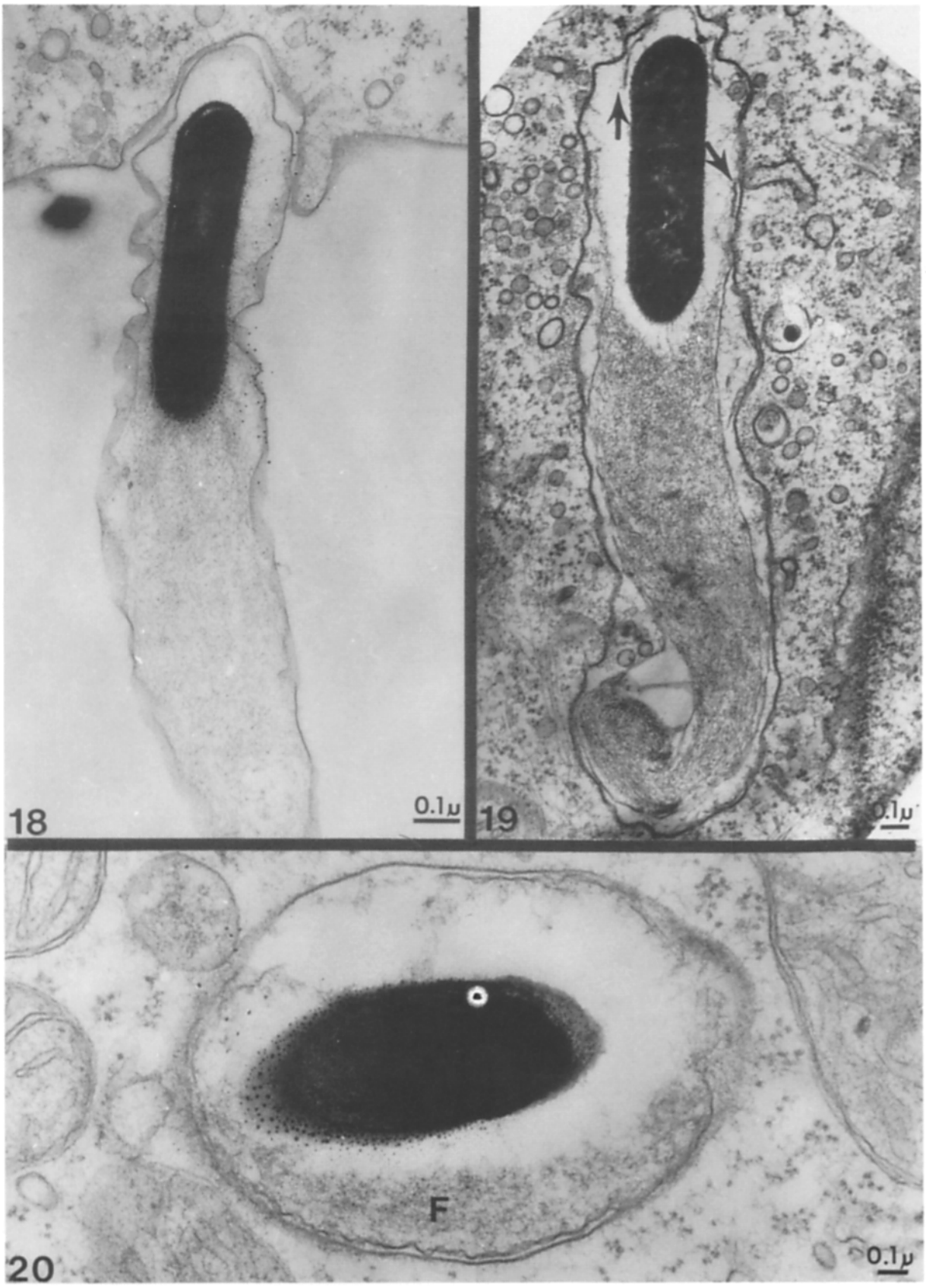
30 Min Infection Followed by 210 Min of Growth

To examine later stages in the multiplication of *Listeria*, their movement to the surface of the host macrophage, and their spread to neighboring macrophages, we infected the macrophages for 30 min, and then washed and incubated the macrophages for 210 min in gentamicin before fixation.

Examination of thin sections of macrophages at low magnification reveals that most of the *Listeria* encountered



Figures 16 and 17. (*16*) Thin section of a portion of the surface of a macrophage infected for 4 h with *Listeria*. These macrophages were fixed in situ in the dish in which they were growing. *a*, Located at the tip of a projection from the macrophage cell surface is a single *Listeria* and behind it a long, fine, filamentous tail. *b*, The fine, filamentous tail at higher magnification. Note that the filaments are randomly oriented relative to each other, some in transverse section (*dots*), others in oblique and longitudinal section. (*17*) Macrophages were infected with *Listeria* for 4 h, then extracted with Triton X-100 and incubated with S1. This section is taken of the same region as Fig. 16. Basal to the *Listeria* at the end of this pseudopod is the fine, filamentous tail whose component filaments are decorated with S1. The small arrow indicates the polarity of several decorated filaments. The large arrow indicates residual membrane that has not been solubilized.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are in the process of division or have recently divided (Fig. 14). This is not unexpected as the intracellular doubling time is ~1 h (Portnoy et al., 1988). They are all surrounded by actin filaments that appear at low resolution as an amorphous layer that separates the *Listeria* from the formed elements of the cytoplasm of the host (Fig. 14). Some newly divided "couples" are in the process of separation, and the mat of filaments begins to elongate away from the former division plane (Fig. 14, *arrows*) as if trying to pull the *Listeria* apart.

The actin filaments then become organized into an array that extends from only one end of the *Listeria* instead of surrounding it. What we see resembles a comet with a long tail. This tail, composed of actin filaments, can exceed 5 μm in length and 1 μm in width (Fig. 15). The actin filaments that compose it are not oriented parallel to each other, forming a compact bundle, but instead are oriented at random. Thus, in a single section, some can extend along the comet axis, others at an oblique angle to the axis, and still others perpendicular to the axis (e.g., cut in cross section). We frequently find these "comets" and their actin tails near the surface of the macrophage. Invariably, the comet proper (*Listeria*) is nearest the cell surface with the actin tail extending towards the cell center. We also find the comets in cell extensions (Fig. 16 *a*); the *Listeria* are always located at the tip of the cell extension with the actin tail extending into the cell proper. Higher resolution micrographs of these cell extensions show that there are few, if any, actin filaments at the tip immediately in front of the *Listeria* comet or along its flanks; instead, they are confined to the tail of the "*Listeria* comet," but are still randomly oriented (Fig. 16, *a* and *b*). This randomness in orientation may be because of inadequate fixation as actin filaments are notoriously difficult to preserve and are severed by the OsO_4 in the fixative (Tilney, 1976; Maupin-Szamier and Pollard, 1978). On the other hand, there are a number of long microvilli (microspikes) extending from the macrophages that are not associated with *Listeria* in which the actin filaments are aligned parallel to one another. Decoration of the actin filaments in these microvilli with S1 of myosin, a procedure that stabilizes actin filaments to osmication, shows that they are parallel to one another. In contrast, those in the comet's tail are randomly oriented (Fig. 17). It is unlikely that the random distribution of actin filaments in the comet's tail is an artefact of fixation, in which case this type of *Listeria* containing cell extension is more akin to a pseudopod where the actin filaments are oriented at random than a microvillus where the filaments form a parallel, crossbridged bundle. Thus, we have two types of cell extensions; microvilli with parallel arrays of actin filaments and pseudopod-like extensions with *Listeria* at the tip and a tail of randomly oriented actin filaments. (The word "pseudopod" is not appropriate because these *Listeria* containing extensions are fingerlike, and not broad as "pseudopod" usually connotes.)

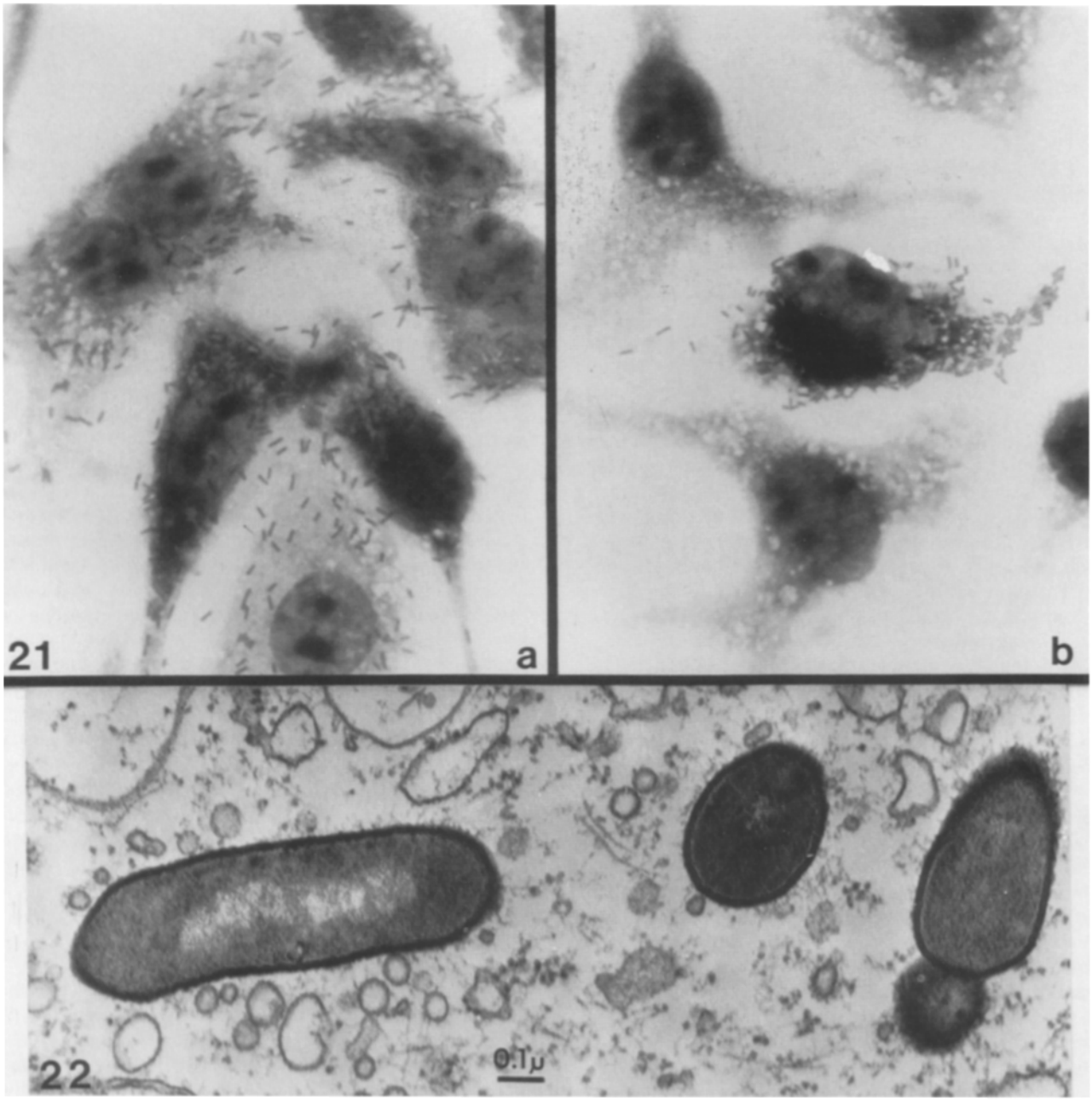
The next step in pathogenesis is the transfer of the *Listeria* from an infected cell to a noninfected neighbor. What we see in our micrographs is that contact is made between the tip of an extension containing a *Listeria* comet and a neighboring macrophage (Fig. 18). There then seems to be phagocytosis of the pseudopod or a portion of it containing the *Listeria* and some of its "tail" by the neighboring macrophage. The newly formed phagosome contains a vacuole within a vacuole; the *Listeria* is enclosed in the plasma membrane of the pseudopod of the donor cell along with actin filaments of the donor cell that in turn is surrounded with membrane from the new host (Fig. 19). What has to follow is the dissolution of both these membranes. This occurs in two stages. First, the vacuolar membrane is dissolved (Figs. 19 and 20), followed by the dissolution of the phagosome membrane; a reaction that occurs at a later time. Thus, this insidious beast has managed to multiply and spread cell-to-cell without leaving the cytoplasm of its host.

The function of the mat of actin filaments that surrounds the *Listeria* and ultimately becomes rearranged to form a comet's tail in the cell extensions must be considered. Do these actin filaments play a role in translocating the *Listeria* to the cell surface of the macrophage and thus present the *Listeria* to a new host? We have some information that they indeed might. This comes from the observation that treatment of infected cells with cytochalasin D not only inhibits spread of *Listeria* from cell to cell but the *Listeria* tend to remain near the cell center, not at the periphery, as if they are incapable of migration (Fig. 21). Furthermore, no comet tails are formed. Examination of thin sections of cytochalasin-treated macrophages in most cases reveals "naked" *Listeria* or *Listeria* devoid of an actin network or a comet's tail. In a few instances, we find some sparse "fuzz" around the *Listeria* (Fig. 22). This does not seem to be actin.

Discussion

In this paper, we have presented a pictorial sequence of the entry, escape into the cytoplasm, replication, migration to the surface, presentation in a pseudopod to a new host cell, phagocytosis of the pseudopod, and dissolution of the encapsulating membranes, and thus infection of a new host by the bacterium, *L. monocytogenes* (see Fig. 23, legend). Up until now only the first step has been documented by EM for *Listeria* (Gaillard et al., 1987), although there is an intriguing report on *Rickettsiae tsutsugamushi*, stating that this bacterium spreads from cell to cell, being phagocytosed by the new host cell (Ewing et al., 1978). Whether *Rickettsiae* associates with actin is unknown.

Figures 18–20. Thin sections of portions of macrophages infected for 4 h with *Listeria* and fixed in situ. (*18*) A projection from an infected macrophage with a *Listeria* at its tip is making contact with the surface of a second macrophage (*top of figure*). Extending behind the *Listeria* is the "tail" of actin filaments described in the previous figure. (*19*) Enclosed within an endosomal membrane of a newly infected macrophage is a *Listeria* and its tail of actin filaments. The *Listeria* and the tail are enclosed by a second membrane that is broken in several places (*arrows*). What has to have happened is that the projection seen in the previous figure has been endocytosed by a second macrophage and the *Listeria* with its hemolysin is dissolving the inner membrane, the former plasma membrane of its old host. (*20*) Higher magnification of an endosome of a newly infected macrophage. Within this endosome is a *Listeria* and a partially dissolved inner membrane (the former host macrophage plasma membrane) and some fine filaments (*F*). Bars, 0.1 μm.



Figures 21 and 22. (*21*) Light micrographs of macrophages infected with *Listeria* for 30 min, then washed and incubated in media containing gentamicin. They were fixed 7.5 h later. *a*, Control. Note that all the cells in the field are infected with *Listeria*. Often the *Listeria* are found near the surface of the cell. *b*, Cytochalasin D, treated for 7 h. Only the central cell is infected with *Listeria*; the other cells are completely negative. Thus, cytochalasin D inhibits spreading. There are ~200 *Listeria* in the central cell. Most are located near the cell center. (*22*) A thin section of a portion of the cytoplasm of a macrophage infected with *Listeria*, then treated with cytochalasin D for 3 h before fixation. Note that there is no mat of actin around the *Listeria* and there are no comet tails. Bar, 0.1 μm.

One purpose in presenting this paper is to begin an in depth description of the cell biology of an intracellular, or more specifically, an intracytoplasmic parasite. We think the choice of *Listeria* is appropriate as it is not tremendously pathogenic and thus too intimidating to encourage further experimentation, it grows efficiently in a macrophage cell line that can be maintained easily in the laboratory, and it is amenable to transposon mutagenesis and plasmid DNA transformation. Furthermore, there is a long history of study of this organism, a model for cell-mediated immunity. The results of this study suggest a cell biological explanation for the absolute requirement of cell-mediated immunity, namely that the bacteria are never extracellular and host humoral antibody would be ineffective.

A priori, no one would have predicted the sequence of cytoskeletal events that occur with *Listeria* infection. More specifically, we might have expected that the comet's tail might be composed of a cross-bridged bundle of actin filaments all parallel to each other so that a microvillus might form or a process like the acrosomal process of invertebrate

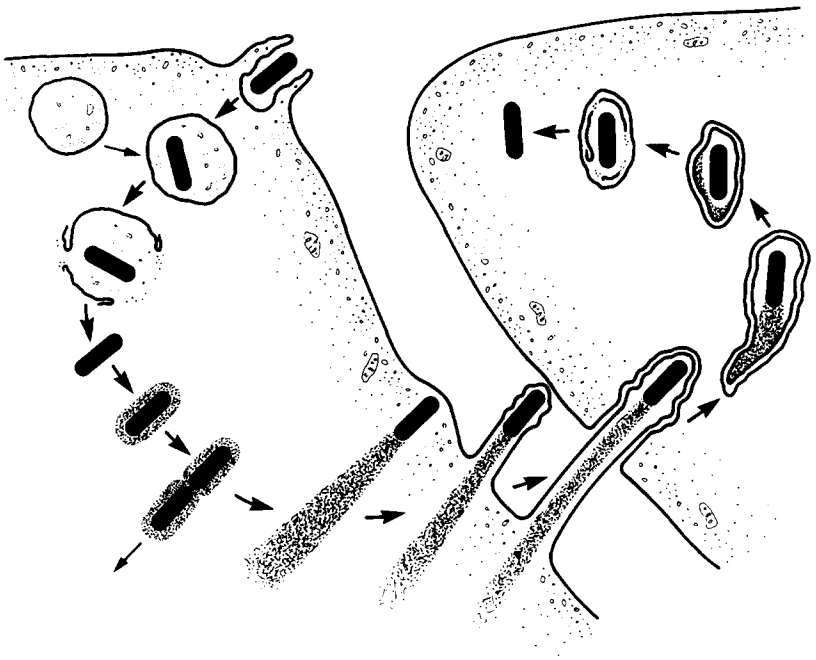


Figure 23. Stages in the entry, growth, movement, and spread of *Listeria* from one macrophage to another. Photographs illustrating all these intermediate stages have been presented in the figures.

sperm would be generated (Tilney, 1985). Instead, what we find is that the actin filaments in the comet's tail seem to be very short and are randomly arranged, yet form a compact cluster that does not associate with the rest of the cytoskeleton of the host macrophage. How this completely novel distribution of actin filaments is generated will have to occupy us in the future.

From published data in the literature, it is reasonable to expect that other intracytoplasmic parasites such as *Rickettsia* and *Shigella* may use the host's cytoskeleton for their own purposes in ways similar to what we describe for *Listeria* (Ogawa et al., 1968; Pal et al., 1989). However, there are probably more intracellular parasites that seem to use entirely different mechanisms or variations on the mechanisms just described to carry out their life cycles on their respective hosts (Moulder, 1985; Edelson, 1982). By studying these "natural variants" we may be able to rapidly find what assembled gene products are necessary, a scenario that can help us learn a great deal about the cell biology of the host macrophage.

As with most scientific studies, a number of questions have arisen from this one. Many of these questions can be answered by looking at living cells as Schaechter et al. started to do in 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.

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