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Survival of intracellular pathogens within macrophages

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Summary. The threat caused by intracellular pathogens increases as conventional drug treatments are less and less effective against a wide range of microorganisms. Understanding the molecular mechanisms used by intracellular pathogens to avoid killing and degradation in their host cells is likely to point at new ways to threat infectious diseases. We discuss some of the strategies used by various microorganisms to avoid killing and degradation in phagolysosomes. Interestingly, it appears that microbes have a lot to teach us about the cell biology and molecular mechanisms of organelle sorting in macrophages.

Keywords: Infectious disease; Phagocytosis; Phagosome fusion; *Leishmania*; Natural-resistance-associated macrophage proteins.

Introduction

Humans and microorganisms have coevolved to benefit from symbiotic relationships. A good example is the interaction existing between humans and the intestinal bacterial flora. However, some microbes pose a real threat to human health and are the causative agents of serious diseases like tuberculosis, salmonellosis, and leishmaniasis to name just a few. The danger posed by microorganisms is even more acute as conventional drug treatments are less and less effective against a wide variety of intracellular pathogens. Thus, in order to develop new ways to fight intracellular pathogens, it becomes imperative to understand at the molecular cell biology level the processes by which microorganisms evade our natural cellular defenses.

Paradoxically, several intracellular parasites can invade and replicate within cells of the immune system specialized in ingesting and killing microorganisms, like neutrophils and macrophages. In these cells, microbes are taken up through phagocytosis and reside in phagosomes, organelles originating from invaginations of the plasma membrane. The newly formed phagosomes will then engage in a complex process of maturation involving the rapid recycling of membrane components to the plasma membrane (Pitt et al. 1992a), binding to and movement along cytoskeletal elements (Blocker et al. 1998), and a series of fusion events with endocytic organelles, giving rise to lysosome-like organelles referred to as phagolysosomes (Desjardins et al. 1994a, Desjardins 1995, Beron et al. 1995). At that point, phagolysosomes are filled with hydrolytic enzymes and toxic oxygen metabolites that contribute to the destruction of microbes. Once degraded, microbial peptides are presented to effector cells of the immune system in order to trigger specific immune responses (Harding et al. 1995, Germain 1995). Unfortunately, intracellular pathogens have evolved strategies to evade killing and degradation within their host cells. Basically, three main survival strategies can be distinguished.

1. Some microorganisms like *Leishmania* spp. amastigotes and *Coxiella burnetii* reside and proliferate with impunity in the harsh environment of mature phagolysosomes.
2. Microorganisms like *Listeria monocytogenes* and *Shigella* spp. avoid their delivery to phagolysosomes by synthesizing lytic enzymes allowing the degradation of the phagosome membrane and their escape to the nutrient-rich cytoplasm.
3. Pathogens like *Toxoplasma gondii*, *Brucella* spp., *Legionella* spp., *Mycobacterium* spp., and the pro-

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mastigote form of *Leishmania* spp. inhibit phagolysosome biogenesis and therefore reside in immature compartments lacking the molecular machinery to kill and degrade pathogens.

In the last few years, several comprehensive reviews have been published on the interaction between pathogens and their host cells (Theriot 1995, Finlay and Falkow 1997, Pizarro-Cerda et al. 1997, Sinai and Joiner 1997). Here, we will put the emphasis on pathogens that survive within macrophages by altering the normal properties of phagosomes and their transformation into phagolysosomes. Particular attention will be given to recent advances in understanding how various pathogens alter the intracellular routing of phagosomes. When possible, we will also discuss mechanisms used by pathogens to alter phagosome properties.

Microorganisms that survive in phagolysosomes

Conceptually, the simplest way for microorganisms to survive in their host cells is to synthesize molecules that allow them to resist the harsh environment of phagolysosomes. In this case, the biogenesis of phagolysosomes is not directly altered. Rather, microbes display sets of virulence factors or molecules conferring resistance to the sustained attack of hydrolytic enzymes and other toxic molecules of their host. This is the strategy used by the amastigote form of *Leishmania* spp. and by *Coxiella burnetii*. Thus, these microbes are internalized and reside in phagosomes that transform normally into phagolysosomes.

Most of our knowledge on the biogenesis of phagolysosomes comes from in vitro studies with inert particles, like latex beads, internalized in cultured macrophages. The use of latex beads to study phagosome biogenesis has several advantages. They are avidly ingested by a variety of cells in culture. They can be covered by different molecules, through opsonization or chemical derivation, to study phagocytosis through various receptors. Furthermore, their great advantage resides in the fact that they have a very low buoyant density, a characteristic that can be used to isolate the phagosomes containing latex beads on simple step sucrose gradients. In this case, the cells are generally fed latex beads for various periods of time, an approach enabling to study the aging or maturation of phagosomes, and are then gently broken to release intact organelles. These organelles can be loaded on a sucrose gradient and phagosomes isolated after a simple 60 min centrifugation in a region of the gradient normally devoid of any organelles. This approach allowed the demonstration that phagosomes sequentially display molecules characteristic of the plasma membrane, early endosomes, late endosomes, and, finally, lysosomes (Lang et al. 1988; Pitt et al. 1992a; Desjardins et al. 1994a, b; Pizon et al. 1994). Among these molecules are a subset of small GTPases of the Rab family, Rab5 and Rab7, involved in the regulation of fusion events among endovacuolar organelles. The sequential appearance and disappearance of these molecules suggested that phagosomes are involved in fusion events with endocytic organelles in a process that could be compared to the one described for the homologous fusion of endosomes (Gruenberg and Maxfield 1995). Indeed, further studies demonstrated that phagosomes fuse preferentially with early endosomes, then with late endosomes, and then with lysosomes (Mayorga et al. 1991, Pitt et al. 1992b, Jahraus et al. 1994, Desjardins et al. 1997). In contrast to the generally accepted concept in which the phagolysosome arises from the fusion of a phagosome with a lysosome, in a single event, we have provided evidence showing that phagolysosome biogenesis is carried on by multiple transient-fusion events between phagosomes and endocytic organelles of various origins (Desjardins et al. 1994a, 1997). This process is referred to as the "kiss and run" hypothesis (Desjardins 1995, Storrie and Desjardins 1996). In vitro studies strongly suggest that cytoskeletal elements are involved in the ability of phagosomes to fuse with endocytic organelles (Blocker et al. 1997). The complexity of the process of phagolysosome biogenesis is attested by the finding that hundreds of polypeptides whose levels are modulated during phagosome maturation are associated with latex-bead-containing phagosomes (Desjardins et al. 1994b, Burkhardt et al. 1995, Scianimanico et al. 1997). Indeed, the identity and function of most of these proteins are still unknown. It is thus not surprising that most of the molecular mechanisms involved in phagolysosome biogenesis are still unknown.

Leishmania spp. (amastigotes)

Parasites *Leishmania* spp. are the causative agents of cutaneous, mucosal and visceral leishmaniasis. The parasites are transmitted to humans in a promastigote form (see below) by sandflies and enter mammalian macrophages apparently through conventional phago-

cytosis, although a recent report indicates that coiling phagocytosis could be involved (Rittig et al. 1998). Early studies have shown that phagosomes containing leishmanial amastigotes display the markers and characteristics of lysosome-like organelles. Indeed, several groups have shown that vacuoles containing *L. mexicana* or *L. amazonensis* are accessible to endocytic tracers and display activity for the late-endosome and lysosome enzyme acid phosphatase (Alexander and Vickerman 1975, Chang and Dwyer 1976, Shepherd et al. 1983, Rabinovitch et al. 1985). Moreover, recent studies have shown that vacuoles housing amastigotes of *L. amazonensis* maintain an acidic pH (Antoine et al. 1990) and that *L. donovani*-containing phagosomes have hydrolases and membrane markers typical of late-endosome and lysosome compartments (Prina et al. 1990, Russell et al. 1992, Lang et al. 1994a). These data are consistent with the proposal that leishmanial amastigotes reside and multiply within phagolysosomes. The molecular machinery allowing amastigotes to survive in phagolysosomes is poorly known. The finding that they do not inactivate phagolysosomal hydrolases suggests that amastigotes are resistant to their action. Unlike the promastigote form, amastigotes do not form a glycocalix capable of protecting them from hydrolases (Pimenta et al. 1991). Instead, they might synthesize proteases involved in the degradation of host molecules in phagolysosomes (Pupkis et al. 1986). An approach to analyze those proteins that are specifically expressed by *Leishmania* spp. within host phagosomes is currently developed in our laboratory to address this issue.

Coxiella burnetii

The rickettsia *Coxiella burnetii* is the causative agent of the Q fever. This bacterium was shown to reside within vacuoles staining for acid phosphatase and containing accumulation of membranes characteristic of late-endocytic or lysosome-like organelles (Burton et al. 1978, Heinzen et al. 1996), and displaying an acidic pH (Maurin et al. 1992). Interestingly, it was shown that *L. amazonensis* amastigotes and *C. burnetii* can be present in the same vacuoles after coinfection, further confirming the ability of these organisms to survive within the same type of phagolysosomes (Rabinovitch and Veras 1996). The mechanisms allowing *C. burnetii* to survive in the hostile environment prevalent in phagolysosomes are poorly understood. Recent reports indicate that *C. burnetii* may produce enzymes eliminating or preventing the formation of oxygen metabolites (Y. Li et al. 1996). Its surface-expressed lipopolysaccharide (LPS) could also be used as a virulent factor (Baca et al. 1994). Interestingly, surface-expressed molecules like LPS, or lipophosphoglycan in the case of *Leishmania* spp., are also used as virulence factors by other microorganisms (see below). Obviously, there is still a lot to learn about the intracellular trafficking of *C. burnetii* and its mechanisms of survival in mammalian cells.

Microorganisms that escape phagosomes

An efficient way for microorganisms to prevent their killing and degradation in phagolysosomes is to escape phagosomes and gain entry to the more cozy environment of the cell cytoplasm. This is the strategy used by microorganisms such as *Listeria monocytogenes*, *Shigella* spp., and *Trypanozoma cruzi*.

Listeria monocytogenes

Listeria monocytogenes is a food-borne pathogen that infects mostly epithelial cells of the intestine causing listeriosis, a disease that can also affect the nervous and reproductive systems (Cooper and Walker 1998). As mentioned above, the main survival strategy used by *L. monocytogenes* is to escape from the phagosome before the environment becomes too hostile and to invade the cytoplasm. It does so by synthesizing listeriolysin O, an enzyme that lyses the phagosomal membrane (Gaillard et al. 1987, Portnoy et al. 1988). The finding that listeriolysin O is activated at an acidic pH (Geoffroy et al. 1987) suggests that phagosomes housing *L. monocytogenes* have to mature to a certain degree allowing the acidification of the compartment. *Listeria monocytogenes* also produces two phospholipases that help degrade the phagosomal membrane (Leimeister-Wacher et al. 1991, Mengaud et al. 1991, Vasquez-Boland et al. 1992). Recent evidence suggests that *L. monocytogenes* might be able to control the degree of maturation of the phagosomes in which they reside (Alvarez-Dominguez et al. 1997). With listeria mutants lacking active listeriolysin, it was recently reported that early-endosomal markers (mannose and transferrin receptors) and proteins necessary for early-fusion events (N-ethylmaleimidine-sensitive membrane fusion protein and soluble N-ethylmaleimidine-sensitive factor attachment protein) accumulate on phagosomal membranes enclosing *L.*

monocytogenes, which are also enriched in Rab5 (Alvarez-Dominguez et al. 1996), a small GTPase known to regulate early-endocytic fusion events (Gorvel et al. 1991, Bucci et al. 1992). In contrast, late-endosomal markers (cation-independent mannose 6-phosphate receptor, CI-M6PR) or lysosomal proteins fail to accumulate on phagosomal membranes, suggesting that the maturation process of listeria-containing phagosomes stops short of the late-endosomal or lysosomal stages. Indeed, escape from a less hostile compartment has the advantage of preventing leakage of hydrolytic enzymes to the cell cytoplasm.

Shigella spp.

Shigella spp. are widely spread in certain tropical areas where it causes severe dysentery. *Shigella* spp. infect epithelial cells of the colon by an entry mechanism involving reorganization of actin filaments and the formation of membrane ruffles (Menard et al. 1996). Like *L. monocytogenes* after their internalization, *Shigella* spp. rapidly escape phagosomes to invade the cytoplasm. This behavior is associated to gene products of the Ipa family, including the *ipaB* gene product that displays a clear lytic activity (High et al. 1992). The same mechanisms appear to be used to invade and escape phagocytic vacuoles of macrophages (Clerc et al. 1987). The rapid lysis of phagosomes containing *Shigella* spp. has, so far, restricted direct study of its composition and properties.

Microorganisms that inhibit phagolysosome biogenesis

The third strategy used by microorganisms to avoid their passage in phagolysosomes is to alter and inhibit phagosome maturation and the acquisition of phagolysosome characteristics. This is the way chosen by pathogens like *Mycobacterium* spp., *Salmonella* spp., *Legionella* spp., *Toxoplasma gondii*, *Brucella* spp., and the promastigote form of *Leishmania* spp. Although these pathogens were initially believed to reside in phagosomes unable to fuse with endocytic organelles and thus preventing phagolysosome biogenesis, it has become apparent in the last few years, that they use several molecular mechanisms to alter phagosome maturation. The current view is that microbes can modify in various ways the intracellular trafficking of phagosomes. Understanding the molecular bases of microbe action on their host cells is likely to yield valuable information on the process of intracellular traffic, allowing to propose alternative biological approaches to fight infectious diseases at a time when drug treatments are failing.

Leishmania spp. promastigotes

In contrast to leishmanial amastigotes, which reside in phagocytic organelles that are able to fuse with lysosomes, *L. donovani* promastigotes are internalized in phagosomes that display poor fusogenic properties toward endocytic organelles (Desjardins and Descoteaux 1997). By a combination of genetic and biochemical approaches it was demonstrated that the lipophosphoglycan (LPG) of this parasite is responsible for the partial inhibition of fusion between phagosomes and endosomes. Indeed, mutants lacking the repeating units of LPG at their surface are present in phagosomes that fuse extensively with endocytic organelles. Because of their poor fusion properties, phagosomes containing wild-type *L. donovani* do not appear to mature properly and acquire less of the late-endosomal and lysosomal marker Lamp1. In contrast, LPG-deficient mutants are present in phagosomes rapidly displaying detectable level of this molecule (Desjardins and Descoteaux unpubl. obs.).

It is not known whether wild-type promastigotes and the repeating-unit-defective mutants use distinct receptors for attachment and entry inside macrophages, a process that could result in the formation of phagosomes with different biochemical composition and fusion properties (Joiner et al. 1990, Small et al. 1994, Finlay and Falkow 1997). However, evidence suggests that LPG alters the properties of the phagosome by a direct and persistent action on its membrane. Russell et al. (1992) have shown that the promastigote-to-amastigote transformation of *L. mexicana* inside phagosomes, which is accompanied by the down regulation of LPG, restores phagosome-endosome fusion.

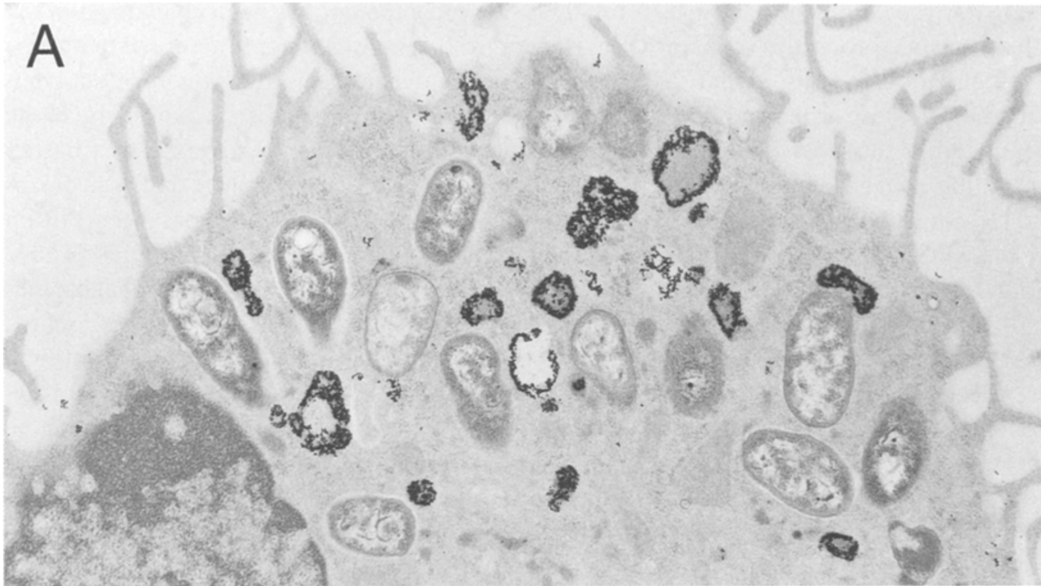
This action of LPG is made possible by its transfer, within minutes, from the promastigote surface to the macrophage membrane at the immediate area of internalization (Tolson et al. 1990). Insertion of LPG in lipid-bilayer membranes results in reduced fusogenic properties possibly by stabilizing the bilayer against the formation of highly curved fusion intermediates (Miao et al. 1995) thought to be required to initiate fusion between phagosomes and endosomes. Full-length LPG is required for this process, as truncated

forms of LPG containing few repeating units are ineffective in modifying the fusogenic properties of membranes (Miao et al. 1995). The observation that *L. donovani* RT5, a mutant expressing truncated LPG with three to five repeating units (McNeely and Turco 1990), is unable to inhibit phagosome-endosome fusion (Desjardins and Descoteaux 1997) supports the requirement for complete LPG molecules.

The partial inhibition of fusion between phagosomes and endocytic organelles induced by *L. donovani* promastigotes has potential importance in the establishment of an effective infection. First, the hydrolase-poor environment encountered in the early phagosome may be more suited to allow the transformation of promastigotes into amastigotes, which are adapted to the conditions prevailing inside phagolysosomes. Second, the limited transfer of hydrolases into the promastigote-containing phagosomes might alter the efficiency with which this cell generates and presents, at its surface, microbial peptides linked to MHC (major histocompatibility complex) class II molecules, a process requiring the hydrolytic action of enzymes present in lysosomes. Several studies have shown that leishmania-containing phagosomes display MHC class II molecules after stimulation with gamma interferon in macrophages (Antoine et al. 1991; Russell et al. 1992; Lang et al. 1994a, b) and in Langerhans cells (Flohe et al. 1997). Antoine et al. (1991) provided evidence that infected macrophages are able to process and present leishmanial antigens together with MHC class II molecules. Furthermore, they showed that macrophages infected with the promastigote form of the parasite were more suited to stimulate T cells than those infected with amastigotes, suggesting that differentiation of *L. amazonensis* was part of the strategy to evade the immune system (Prina et al. 1996). The finding that *L. amazonensis* can internalize and degrade some of its host MHC class II molecules also indicates a potential way by which the parasite could circumvent its host immune system (De Souza Leao et al. 1995).

Mycobacterium spp.

Mycobacterium spp. enter macrophages through phagocytosis and reside in compartments that fail to fuse with lysosomes (Fig. 1) (Armstrong and Hart 1971, de Chastellier et al. 1993, Xu et al. 1994, Clemens and Horwitz 1995, Barker et al. 1997). However, the idea that mycobacterium-containing phagosomes are totally fusion incompetent has been challenged in the past few years. It is now commonly accepted that these phagosomes are dynamic and fusion-competent compartments. Using electron microscopy and pulse chase experiments with horseradish peroxidase, de Chastellier et al. (1995) have clearly shown that transfer of horseradish peroxidase to phagosomes occurs from early endosomes. Clemens and Horwitz (1995) have shown that *M. tuberculosis*-containing phagosomes acquire endosomal markers like CD63, Lamp1, Lamp2, and cathepsin D, albeit to a lesser extent than fully fusogenic phagosomes containing dead mycobacteria or latex beads. Moreover, Russell et al. (1996) have elegantly demonstrated that phagosomes housing *M. tuberculosis* are accessible by glycosphingolipids from the plasma membrane. Further investigations from this laboratory confirmed that mycobacterium-containing phagosomes display a composition similar to that of early endosomes and may stop their maturation at a transitional state in phagolysosome biogenesis (Sturgill-Koszycki et al. 1996). This study has also shown the presence of endosomal markers such as Lamp1, the immature form of cathepsin D, and cathepsin B and L on these phagosomes. Since cathepsins are normally processed to their mature forms in lysosomes, these results confirm that mycobacterium-containing phagosomes fail to transform into phagolysosomes. Further similarities with early-endocytic organelles have been described. Mycobacterium-containing phagosomes are accessible to exogenous transferrin (Clemens and Horwitz 1996) and are enriched with the small GTPase Rab5, but devoid of the late-endosome marker Rab7 (Via et al. 1997, Deretic et al. 1997). Since Rab5 is known to regulate early endocytic events whereas Rab7 is involved in late events (Chavrier et al. 1990, Gorvel et al. 1991, Méresse et al. 1995, Feng et al. 1995), these results suggest that mycobacterium-containing phagosomes display the machinery required to engage in fusion events with Rab5-enriched early endosomes, but lack molecules allowing subsequent fusion with late-endocytic organelles. This lack of fusion with late-endocytic organelles is likely to explain the absence of proton pump ATPases from these phagosomes and their improper acidification (Sturgill-Koszycki et al. 1994). In turn, lack of acidification may interfere with the recruitment of key fusogenic molecules from the host cell cytoplasm (Aniento et al. 1997). Systematic characterization of the composition of mycobacterium-containing phagosomes by high-resolution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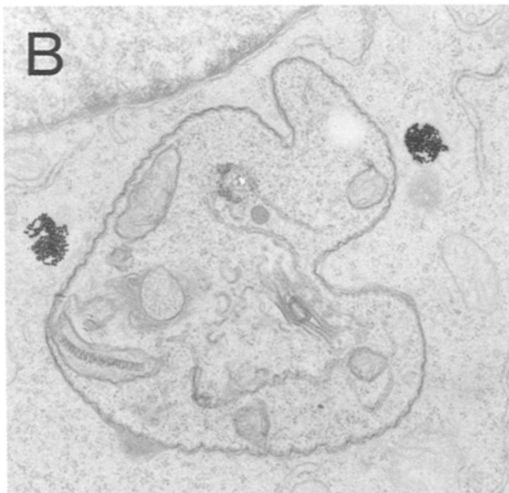
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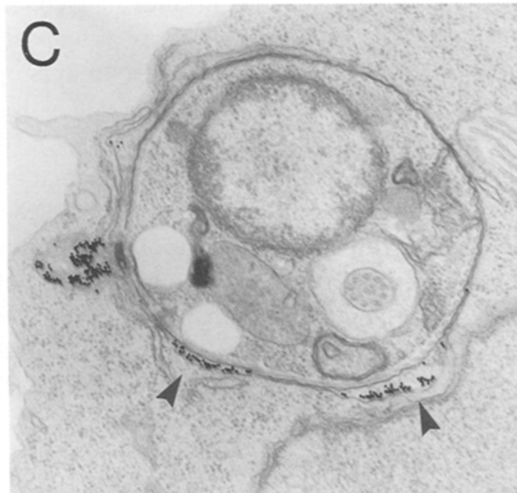
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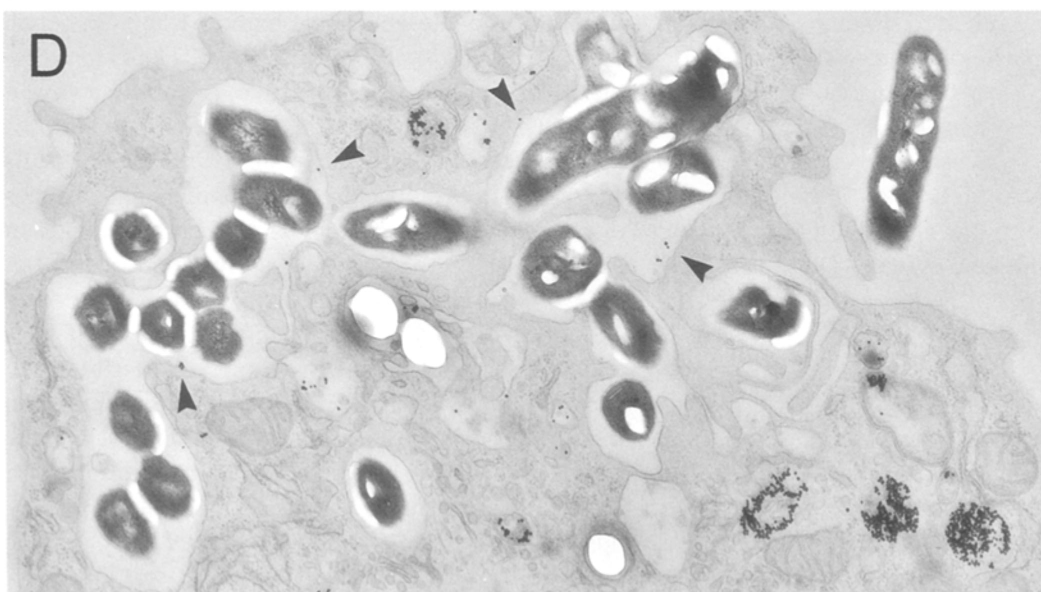
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two-dimensional gel electrophoresis might indicate the nature of proteins involved in the alteration of their fusion properties (Sturgill-Koszycki et al. 1997, Hasan et al. 1997).

The mechanisms used by *Mycobacterium* spp. to inhibit phagosome-lysosome fusion are still poorly understood. Early studies indicated that it may involve the production of ammonia interfering with movement of lysosomes and their potential interaction with phagosomes (Gordon et al. 1980, Hart et al. 1983). Virulence factors such as glycolipids present at the bacterial surface (Ehlers and Daffe 1998), in contact with the inner phagosomal membrane after internalization, may also affect the properties of phagosomes.

Salmonella spp.

Members of the genus *Salmonella* infect nonphagocytic cells of the intestinal epithelium by mechanisms involving ruffling of the plasma membrane (Finlay 1994). They also enter macrophages either through conventional phagocytosis, which gives rise to closely fitting phagosomes, or through induction of macropinocytosis, which gives rise to spacious phagosomes (Alpuche-Aranda et al. 1994). Spacious vacuoles are thought to enhance intracellular survival by diluting toxic compounds present in phagolysosomes. The subsequent trafficking and properties of these vacuoles have been the subject of intense research yielding conflicting results. It was reported that, in macrophages, *S. typhimurium* reside in compartments that fuse poorly with lysosomes (Ishibashi and Arai 1990, Buchmeier and Heffron 1991), although the latter study showed a phagosome-lysosome fusion level of about 40% in peritoneal and J774 macrophages. Both studies indicated that the surface-expressed LPS was not involved in this inhibition of fusion. A partial inhibition of fusion was also shown to occur in epithelial cells (Garcia-del Portillo and Finlay 1995). In this case, around 30% of the phagosomes containing *S. typhimurium* also contained horseradish peroxidase previously internalized and chased to lysosomes. These phagosomes were shown to display lysosomal glycoproteins but to lack M6PR and high level of cathepsin D. Similar patterns of labeling were reported in macrophages (Rathman et al. 1997). These two studies suggest that *S. typhimurium* is internalized in phagosomes that interact poorly with M6PR-positive compartments. In contrast, a recent study reported the complete fusion of lysosomes with phagosomes containing *S. typhimurium* indicating its ability to survive within mature phagolysosomes displaying Lamp1 and the lysosomal enzyme cathepsin L (Oh et al. 1996). This discrepancy was suggested to be caused by the use of peritoneal macrophages by Oh et al. (Rathman et al. 1997).

Although further studies are required to define clearly the intracellular routing of salmonella-containing phagosomes, it is apparent that these phagosomes engage in a maturation process resulting in their rapid acidification (Alpuche-Aranda 1992, Rathman et al. 1996), an essential step for the activation of bacterial survival genes.

Legionella pneumophilia

Legionella pneumophilia, the causative agent of a pneumonia known as Legionnaires' disease, is a facultative intracellular parasite that infects alveolar macrophages (McDade et al. 1977). It enters its host cells through a specialized form of phagocytosis referred to as coiling phagocytosis (Horwitz 1984) and resides in phagosomes that fail to acidify and fuse with lysosomes (Horwitz 1983a, Horwitz and Maxfield 1984). Following entry, phagosomes sequentially associate with smooth vesicles, mitochondria, and endoplasmic reticulum (Horwitz 1983b, Swanson and Isberg 1995). Phagosomes eventually relocate near the nucleus where they appear as vacuoles covered with ribosomes due to their close association with the rough endoplasmic reticulum (Horwitz 1983b). These final compartments, referred to as replicative phagosomes, display similarities with autophagic vacuoles (Swanson and Isberg 1995). They lack transferrin receptors and fail to acquire late-endosomal and lysosomal markers like Lamp1, Lamp2, CD63, and cathepsin D (Clemens and Horwitz 1995). These results indicate that maturation of these vacuoles toward phagolysosomes is

Fig. 1 A–D. Interaction of endocytic organelles with phagosomes containing various microorganisms. Phagosomes containing brucellas (**A**), the promastigote *Leishmania donovani* (**B**), or mycobacteria (**D**) interact poorly with endocytic organelles. As a result, all these microorganisms reside in phagosomes that do not transform into phagolysosomes. In contrast, the amastigote form of *Leishmania donovani* (**C**) resides in phagosomes that fuse with endocytic organelles and display the characteristics of phagolysosomes

inhibited early after phagocytosis, possibly because of the inhibition of phagosome-endosome fusion.

Recent studies from various laboratories have identified a family of genes involved in bacterial virulence. These genes are referred to as *icm/dot*, for "intracellular multiplication" and "defective in organelle trafficking" (see Segal and Shuman 1998, Kirby and Isberg 1998). The *dotA* gene was shown to encode an inner-membrane protein required for phagosome-lysosome fusion inhibition (Roy and Isberg 1997, Roy et al. 1998). The Icm/Dot complexes are capable of forming pores into eukaryotic membranes and transferring plasmid DNA from one cell to another (Kirby et al. 1998, Vogel et al. 1998). Therefore, it is possible that the Icm/Dot system functions by secreting macromolecules into the cell cytoplasm that could prevent phagosome-lysosome fusion. Such macromolecules, proteins for example, could be inserted in the phagosomal membrane via the pore formed by the Icm/Dot system and alter its fusion properties (Segal and Shuman 1998). Other genes of the *icm/dot* family, *dotH*, *dotI*, and *dotO*, have been recently identified as being essential for growth and evasion of the endocytic pathway (Andrews et al. 1998). Although their function is still unknown, bacterial mutants for these genes are found in phagosomes positive for Lamp1, suggesting a defect in subverting the normal intracellular trafficking.

Brucella spp.

Bacteria of the genus *Brucella* cause a disease known as Malta fever. They infect mostly cells of the reticuloendothelial system, particularly macrophages, but can also infect nonphagocytic cells. Inhibition of phagosome-lysosome fusion also seems to be the mechanism involved in brucella survival within their host cells (Pizarro-Cerdá et al. 1998a). Indeed, a recent study, involving virulent and attenuated strains of *Brucella abortus*, reported the inhibition of phagosome-lysosome fusion by virulent strains in nonprofessional phagocytic HeLa cells while nonvirulent strains failed to induce such inhibition (Pizarro-Cerdá et al. 1998a). However, both virulent and avirulent brucella strains reside in phagosomes displaying similarities in composition to early endosomes, as shown by the presence of EEA1 and Rab5, two early-endosomal markers detected on phagosomes shortly after infection (Pizzaro-Cerdá et al. 1998b). Furthermore, phagosomes containing virulent *B. abortus* do not acquire late-endosomal markers like the cation-independent and the cation-dependent M6PR, the small GTPase Rab7, and the lysosomal protease cathepsin D. However, they gradually accumulate Lamp1 and Lamp2. These results suggest that *B. abortus* inhabits compartments transiently capable of fusion with early endocytic organelles while unable to fuse with late compartments, but somehow acquiring Lamp1 and Lamp2 from sources other than lysosomes.

In addition to fusion inhibition, *B. abortus* has the unique characteristic of being targeted to the endoplasmic reticulum by using the host cell autophagic machinery (Pizzaro-Cerdá et al. 1998b). Indeed, the Lamp1-positive, cathepsin D-negative compartments, in which brucellas reside, resemble autophagic vacuoles as observed by electron microscopy (Pizarro-Cerdá 1998a). These autophagic vacuoles or autophagosomes are thought to stem from regions of the rough endoplasmic reticulum depleted of ribosomes (Dunn 1990a, b). The presence of Sec61β, an endoplasmic reticulum translocator protein, at the brucella-containing phagosomal membrane and the accumulation of monodansylcadaverin, a fluorescent marker previously shown to label autophagosomes specifically (Biederbick et al. 1995), support the idea of autophagosomes as the replicative compartment for *B. abortus* (Pizzaro-Cerdá et al. 1998b). The association of *B. abortus* to the endoplasmic reticulum would provide a favorable environment for intracellular replication. Although the previous results were obtained with nonphagocytic cells, recent evidence shows that a similar intracellular trafficking may be involved in professional phagocytes (Pizzaro-Cerdá unpubl. obs.).

Beside the unique trafficking pathway, *B. abortus* may survive with the help of other virulence factors. The surface glycoconjugate, LPS, may be one of them. Indeed, pathogenic strains produce a smooth LPS and are resistant to intracellular killing, while rough strains produce an LPS lacking O-chain and are not as resistant to killing (Kreutzer et al. 1979, Riley and Robertson 1984). The potential involvement of LPS in fusion inhibition for brucellas has not been investigated yet, but it would be interesting as LPG of leishmanias has been shown to be responsible for fusion inhibition (Desjardins and Descoteaux 1997). Another role for LPS was found by Martinez de Tejada et al. (1995), who demonstrated that resistance to oxygen-independent mechanisms like bactericidal cationic peptides was linked to surface-expressed LPS. Both rough and

smooth LPS contribute to the resistance, but smooth LPS seems to be more efficient.

Besides LPS, there are other factors that contribute to resistance of *B. abortus* to killing, namely the stress response proteases of the high-temperature requirement A (HtrA) family that represents a defense system against oxidative killing (Elzer et al. 1996). Evidence implicating the HtrA family as a defense against killing were also found for other bacteria including *S. typhimurium* (Bäumler et al. 1994, Boucher et al. 1996, Johnson et al. 1991, S. Li et al. 1996). All of these elements contribute to the survival and replication of the parasites.

Toxoplasma gondii

Toxoplasma gondii is the etiological agent of toxoplasmosis, a disease that can affect many organs and is encountered mainly in immunocompromised individuals. The parasites enter macrophages and other nucleated cells through an active process that differs from conventional phagocytosis. Internalization is driven by the parasite's ATP and actin cytoskeleton and does not depend on host cell endocytic machinery (for a detailed review, see Sinai and Joiner 1997). As a result, *T. gondii* reside in parasitophorous vacuoles incapable of fusion with host endocytic organelles (Jones and Hirsch 1972, Sibley et al. 1985, Joiner et al. 1990). The parasitophorous vacuole has little in common with normal phagosomes. It arises from a mechanism of entry that does not involve the specific receptors required for phagocytosis (Morisaki et al. 1995, Dobrowolski and Sibley 1996), and it excludes host plasma membrane-associated proteins (de Carvahlo and de Souza 1989, Pacheco-Soares and De Souza 1998). This exclusion may account for the fusion incapacity of the vacuole which apparently lacks the molecular machinery required for fusion. However, when parasites enter macrophages through receptor-mediated phagocytosis, after opsonization with immunoglobulin G, the vacuoles formed are fusion competent and mature normally into phagolysosomes (Joiner et al. 1990, Mordue and Sibley 1997). This suggests that the mechanism of entry determines the fate of parasites within macrophages. This is further demonstrated by the finding that killing of parasites inside phagosomes, after entry, does not restore the fusion competence of vacuoles (Joiner et al. 1990).

Immunocytochemical studies indicate that *T. gondii*-containing vacuoles lack detectable levels of cell surface markers, transferrin receptors, Igp, and M6PR (de Carvalho and de Souza 1989, Joiner 1992, Joiner et al. 1990, Mordue and Sibley 1997), indicating that traffic of these vacuoles does not follow the phagolysosome pathway. Rather, they associate with the endoplasmic reticulum and mitochondria (Sinai et al. 1997). The significance of this association is unknown. It could serve as a nutrient delivery system or could provide building material to expand the vacuole membrane as parasites replicate. The role of endoplasmic reticulum and mitochondria in lipid synthesis goes along with this idea (Trotter and Voelker 1994, Vance and Shiao 1996).

Resistance to infection

It is well documented that killing of intracellular parasites, for instance mycobacterium to name just one, is correlated with the level of activation of macrophages by various cytokines, including gamma interferon (Rook et al. 1986, Flesch and Kaufmann 1987, Denis 1991, Via et al. 1998, Schaible et al. 1998). However, besides the ability to become activated and more efficient at killing, some populations of macrophages have a genetically based natural resistance to intracellular infection. A well-known genetic determinant controlling infection is the *Nramp1* gene product which confers resistance to a series of unrelated intracellular pathogens including *Mycobacterium* spp., *L. donovani*, and *S. typhimurium* (Plant and Glynn 1976, Bradley 1977, Gros et al. 1981). Interestingly, the strategy used by those three microbes to survive within their host cells is to inhibit to different extents phagosome-endosome fusion. This suggested that Nramp1 might play its role within phagosomes. The recent findings that Nramp1 is expressed only in polymorphonuclear leukocytes and macrophages and is recruited to the membrane of phagosomes confirm this hypothesis (Cellier et al. 1997, Gruenheid et al. 1997). The recruitment of Nramp1 to the phagosomal membrane has been shown to follow kinetics similar to that of Lamp1 (Gruenheid et al. 1997). Interestingly, phagosomes containing one of the three pathogens whose infection is controlled by Nramp1 also acquire Lamp1 at some point during their maturation. In contrast, *Listeria* spp. and *Legionella* spp., which are not under genetic control of Nramp1, fail to acquire this marker (see above).

Nramp1 is a member of a family of metal ion transporters (Cellier et al. 1995, 1996), although its sub-

strate is still unknown. Another member of the family, Nramp2, has been shown to be an iron transporter (Fleming et al. 1997; Fleming et al. 1998). Also, recent evidence indicates that a yeast Nramp homologue, SMF1, acts as a Mn^{2+} transporter (Supek et al. 1996). On the basis of these results, it is possible that Nramp1 may work by removing ions essential for pathogen survival within phagosomes, making the intraphagosomal environment inhospitable to pathogens. For example, Mn^{2+} is an essential cofactor of superoxide dismutase. This enzyme is expressed by *L. donovani*, *M. tuberculosis*, *M. bovis*, and *S. typhimurium* (Leid et al. 1989) and is involved in the neutralization of toxic oxygen metabolites in the phagosome. Thus, by removing such ions from phagosomes, Nramp1 might compromise the ability of microorganisms to sustain attack by toxic metabolites. An Nramp homologue has also been found in *Mycobacterium* spp. (Cellier et al. 1996). In this case, pathogen and host cells might compete for a particular ion, stressing the importance of metal ion homeostasis inside phagosomes for pathogen survival (Agranoff and Krishna 1998).

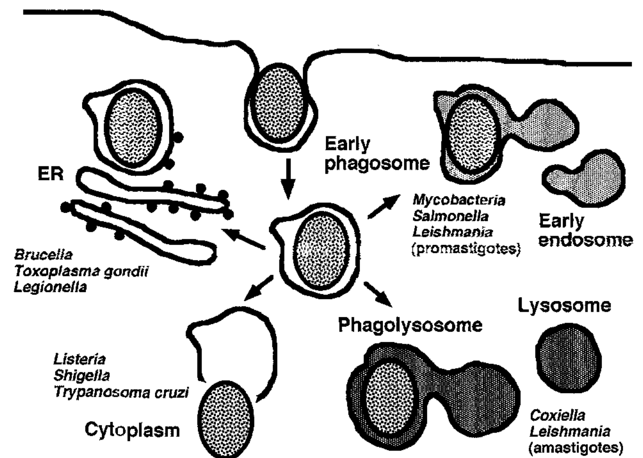

Fig. 2. Intracellular sorting of phagosomes containing various microorganisms. Although they all originate from the plasma membrane, phagosomes containing various microorganisms will transform in organelles displaying characteristics of early endosomes, late endosomes (or lysosomes), or even the endoplasmic reticulum. The molecular mechanisms involved in the transformation of these phagosomes are poorly known

Future perspectives

During the last few years, several laboratories have contributed to the understanding of how intracellular pathogens can evade human natural defenses to replicate with impunity within their hosts. A clear image is starting to emerge regarding the intracellular trafficking of phagosomes containing various microorganisms. Indeed, more than one way is used by microbes to bypass the transfer to the harsh environment encountered in phagolysosomes. This implies that mechanisms exist to transform phagosomes originating from the plasma membrane into a variety of compartments that display similarities with either the endoplasmic reticulum or early endosomes and late endosomes (or lysosomes) (Fig. 2). Most of the molecular details, molecules and mechanisms, governing these transformations, on both the host and parasite, are unknown. The use of an approach using tools from microbiology, cell biology, and molecular genetics promises to give new insights into the complex interaction between microorganisms and their host cells. For example, in the last few years, we have initiated a systematic characterization of the proteins associated with phagosomes. Using high-resolution two-dimensional gel electrophoresis, we have shown that highly enriched phagosome preparations contain hundreds of polypeptides (Desjardins et al. 1994b, Burkhardt et al. 1995). Although most of these proteins have not yet been characterized, we have recently started to use mass spectrometry to identify them. This approach will allow us to generate a two-dimensional gel database that will display the positions of these hundreds of phagosome proteins. This should then enable to follow changes in the composition of phagosomes formed in cells by various microorganisms and to identify some of the components of the machinery governing phagolysosome biogenesis.

Understanding the machinery involved in phagosome transformation could potentially lead to new therapeutic approaches, based on a modulation of the trafficking of microbes. Intracellular pathogens that normally are capable of evading the phagolysosome could then be redirected towards this compartment allowing efficient antigen processing and presentation to occur. In other words, there is a hope of developing new vaccination strategies.

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