Invited Review

When intracellular pathogens invade the frontiers of cell biology and immunology

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Summary. Cellular microbiology has recently been described as a new discipline emerging at the interface between cell biology and microbiology (Cossart et al., 1996). Many microbial pathogens can enter eukaryotic cells and live intracellularly either inside vacuoles or in the cytoplasm. The different steps during the invasion process are on the way of being dissected at the molecular level revealing new insights in basic cellular functions. Indeed, bacterial pathogenesis can help us to better understand the dynamics of cell cytoskeleton, intracellular membrane traffic and signal transduction events. The recent advancements in the field of microbial pathogenesis are creating a new cross-talk between cell biologists, microbiologists and immuno-logists. In this review, the different strategies used by several pathogens are presented and the mechanisms elaborated by host cells from the immune system to eliminate the parasites discussed.

Keywords: Microbial pathogens, Intracellular parasitism, Membrane traffic, Vaccines, Phagocytosis, Cellular microbiology

Introduction

Evolution shows us that there has been long term cognate interaction between microbial prokaryotic and eukaryotic cells. While some of these interactions have evolved in symbiosis, several microorganisms from diverse phylogenetic origins have derived into intracellular pathogens. Bacteria in particular have developed remarkable biochemical diversity and could exploit any biologically possible nest. The plasma membrane surface, the cytoplasm or intracellular compartments are locations where microbes can actively transit or reproduce by interacting with the eukaryotic cellular environment. This behaviour can lead to cellular or tissue damage, depending both on the capacity of the bacteria to defeat the host defensive mechanisms and on the power of host reactions to eliminate the bacteria. Invasive bacteria elicit specific responses from defined cells of the immune system which often are the primary targets of microbial assault. This review focuses on the mechanisms used by microbial pathogens to enter, transit, reproduce or escape from animal host cell compartments.

Mechanisms of entry

To gain access into the cytoplasmic compartments, some parasites have developped active invasion mechanisms which involve expression of bacterial proteins specialized in the entry into the host cell. Many of the details regarding bacteria entry have been obtained from the structural, genetic and molecular analysis of pathogenic bacteria such as Yersinia sp., Shigella flexneri, and Listeria monocytogenes (Hale, 1991; Sansonetti, 1991; Falkow et al., 1992; Galan, 1994; Theriot, 1995).

Yersinia

One of the best studied systems is that of Yersinia pseudotuberculosis and its outer-membrane protein, invasin, the 103 kDa product of the inv gene (Isberg and Falkow, 1985; Isberg et al., 1987; Isberg, 1991). This surface molecule promotes entry by a zipper mechanism which involves the invasin binding to multiple β1 chain integrin receptors on the host cell plasma membrane (Isberg and Leong, 1990; Leong et al., 1990; Isberg and Tran Van Nhuie, 1995; Swanson and Baer, 1995). Since the integrins are present both in professional and in non professional phagocytic cells, the invasin-mediated internalisation into epithelial cells would be similar to that observed in macrophages. The interaction between invasin and β1 integrin requires the carboxyl terminal 192 aminoacids of the bacterial protein (Leong et al., 1990). A second mechanism of entry to be used by Yersinia also involves the interaction between integrin receptors and the surface protein YadA encoded by the 70 kb virulence-associated plasmid (Bliska et al., 1993; Yang and Isberg, 1993). In addition to these two
pathways, the 17 kDa Yersinia Ail protein was shown to promote bacterial uptake from a wide variety of mammalian cell lines (Miller et al., 1990; Bliska and Falkow, 1992). The expression of these three independent proteins involved in invasion is tightly regulated by temperature: expression of invasion is highest at ambient or lower temperature before bacterial infection occurs (Isberg and Leong, 1988), whereas Ail protein expression mainly initiates when the bacteria are growing within the host. These observations suggest that the three pathways work sequentially during Yersinia infection.

**Shigella**

*S. flexneri* has the ability of being internalized by cells that are not phagocytic in vitro. In vivo, *S. flexneri* is known to enter epithelial cells through the basolateral membrane (Mounier et al., 1992). Several proteins of the *ipa* operon are required to allow *Shigella* entry into epithelial cells (Andrews et al., 1991; Venkatesan et al., 1992). They require a specialized machinery (encoded by the *msi* and *spa* operons) to be secreted by the bacteria. Mutants for the IpaB, IpaC and IpaD proteins are unable to enter cells cultured in vitro, although the bacteria can adhere to the cell surface (Menard et al., 1993). Within the bacterial cytoplasm, IpaB and IpaC are prevented from associating with each other by the IpgC product that acts as a molecular chaperone for these two proteins. After secretion, IpaB and IpaC associate in a soluble complex (Ipa complex) responsible for parasite-mediated phagocytosis (Menard et al., 1996). Ipa complex induces cytoskeleton rearrangements in the host cell (Menard et al., 1994). However, the host cell receptors for the Ipa proteins remain to be characterized.

**Listeria**

In the case of *L. monocytogenes*, an 80 kDa bacterial surface protein called internalin is required for the entry of *Listeria* into epithelial cells (Gaillard et al., 1991). Internalin is encoded by *inl A* gene: the first region is made up of fifteen 22 amino acid leucine-rich repeats. The second region is formed by three consecutive repeats, two of 70 amino acids and one of 49 amino acids. In the carboxylic part of internalin, sequence homologies with other surface proteins of gram-positive cocci suggest that internalin is anchored to the peptidoglycan by covalent linkage via a threonine residue of the conserved motif LPXTG (Fischetti et al., 1990; Schneewind et al., 1995). In agreement with these structural features, most of internalin is detected at the bacterial surface even if a fraction can be secreted (Drani et al., 1993). Recently, it has been demonstrated that the internalin receptor is the calcium-dependent adhesion molecule E-cadherin that normally plays a role in maintaining the tissue architecture (Mengaud et al., 1996). E-cadherin is found in the nervous system, and skeletal and cardiac muscles, and seems to be preferentially expressed in epithelial tissues at the basolateral membrane of enterocytes (Hermiston and Gordon, 1995), suggesting that *L. monocytogenes* may penetrate the intestinal barrier by the basolateral pole of epithelial cells (Gaillard and Finlay, 1996).

**Signal transduction and cytoskeletal modifications upon entry**

Recent studies on the pathogenic mechanisms of several intracellular bacteria have revealed novel strategies of infection that involve signal transduction events. The best known bacterial models that exploit the modifications of protein phosphorylation activity of the host cell as a part of strategy to penetrate within eukaryotic cells are those of *Yersinia, Salmonella* and *Shigella*.

**Yersinia**

The clustering of integrins promoted by *Yersinia* invasins (Isberg, 1991) triggers tyrosine protein kinase (TPK) activity and seems to be the first step during bacterial uptake. Indeed, it has been shown that TPK inhibitors block the invasion process (Rosenshine et al., 1992). In a second step, interactions between bacteria and host cells lead to the expression of Yop proteins, a group of molecules that interfere with several key host cell functions like tyrosine phosphorylation, actin microfilament stability and G protein-associated receptor stimulation. The identification of YopH as a protein tyrosine phosphatase led to a controversial interpretation of *Yersinia* invasion mechanisms. Indeed, upon integrin receptor-mediated interaction, *Yersinia* promotes signal transduction mechanism in host cell cytoplasm by activation of the protein tyrosine kinases involved in both bacteria entry and host defense responses. These two different activities were observed in macrophages as well as in epithelial cells (Bliska and Falkow, 1992; Bliska et al., 1993; Rosenshine and Finlay, 1993). In contrast, YopH acts as a phosphatase avoiding the entry of *Yersinia* (antiphagocytic activity) but inhibiting at the same time the host response against the bacteria. It may well be that Yops belong to a set of bacterial regulator proteins that control host cell responses upon bacterial entry.

**Salmonella**

The invasion of *S. typhymurium* also involves the subversion of the host cell signaling pathways. *S. typhymurium* activates the epidermal growth factor (EGF) receptor tyrosine kinase activity (Galan et al., 1992), which regulates the intracellular calcium concentration, and consequently cytoskeleton rearrangements. It has also been found that *S. typhymurium* could activate the mitogenic activating protein kinase (MAPK), but in an invasion-independent manner (Rosenshine et al., 1994). The direct
consequences of the signaling transduction cascades induced by Salmonella are still unknown. They could be the initial stimuli for the morphological rearrangements that later correlate with Salmonella invasion. Indeed, it has been observed that interactions between Salmonella and intestinal cells first cause dissolution of microvilli and then provoke profound cytoskeletal rearrangements and membrane ruffles. Pretreatment of epithelial cells with the microfilament inhibitor cytochalasin D blocks bacterial internalization but does not affect the recruitment of polymerized actin, tubulin, tropomyosin, talin and α-actinin beneath adhered bacteria. This suggests that Salmonella bound at the cell surface triggers cytoskeletal rearrangements which are necessary for bacterial entry (Finlay et al., 1991). After internalization, these filamentous components return to their initial state. In contrast to the membrane ruffles generated by EGF receptor interaction, those induced by Salmonella are rac, ras, and rho small GTPases independent (Jones et al., 1993).

**Shigella**

Upon contact with epithelial cells and secretion of the Ipa proteins, Shigella induces activation of the proto-oncogene protein pp60^c-src^ and subsequent tyrosine phosphorylation of its substrate, cortacin (Dehio et al., 1995). Both of these proteins accumulate in the entry structures and at the periphery of the phagosome membranes, where pp60^c-src^ appears to concentrate. This suggests that pp60^c-src^ may play a role in the formation of membrane ruffles and in other cytoskeletal rearrangements observed during Shigella invasion.

For bacterial entry, there is an absolute requirement of actin polymerization in the host cell (Fig. 1), as shown by the complete inhibition of cell invasion after treatment with cytochalasin D (Clerc and Sansonetti, 1987). The bacterium induces the formation of actin nucleation zones that are disseminated beside or under the parasite-contact sites with the cell membrane, while the subcortical actin filaments remain intact (Fig. 1B). Later on, the nucleation zones push up cellular protrusions that rise beside the entering bacterium, being sustained by tightly bundled long actin filaments organized in parallel orientation, where the actin-bundling protein plastin concentrates (Fig. 1C). Finally, the cellular projections coalesce above the bacterial body, leading to its internalization (Adam et al., 1995).

**Toxoplasma gondii and Trypanosoma cruzi**

As opposed to Yersinia and Salmonella, Toxoplasma gondii is a protozoan parasite that actively invades its host cells rather than entering by phagocytosis (Sibiecy, 1995). The invasion is dependent on parasite actin filaments but not on the host-cell cytoskeleton. In the presence of cytochalasin D penetration of the pathogen is inhibited in cytochalasin D-resistant host cells, and mutations, rendering the protozoans resistant to cytochalasin D, restituted the invasion capability (Dobrowolski and Sibley, 1996). The invasion process does not induce membrane ruffling, actin microfilament reorganization or tyrosine phosphorylation in human fibroblasts (Morisaki et al., 1995).

Another protozoan parasite that uses an unusual invasion mechanism is Trypanosoma cruzi. Initial scanning electron micrographs showed that the pathogen was able to penetrate into epithelial cells with minimal disturbance of the plasma membrane of host cells (Schenkman et al., 1988). Later, it was shown that invasion occurred even in the presence of high concentrations of cytochalasin D, and that internalized trypanosomes were not surrounded by actin (Schenkman et al., 1991). Tardieux et al. (1992) demonstrated that lysosomes aggregate at the site of trypanosomes attachment and fuse with the vacuole at early stages of formation (Andrews, 1995).

**Intracellular trafficking**

Pathogens enter mammalian cells by phagocytosis or parasite-directed invasion. In either case, internalized bacteria are encircled by a membrane-surrounded vacuole. Intracellular pathogens then follow the phagocytic route or alter it for their own benefit. Many intracellular pathogens have become adapted to live within membrane-bound compartments (Salmonella, Coxiella, Chlamydia, Legionella, Bartonella, Mycobacterium and Brucella) while others lyse the vacuolar membrane to enter the cytosol (Rickettsia, Shigella and Listeria). We will concentrate on some of the best known strategies followed by these two groups of pathogenic bacteria.

**Lysis of membrane-bound compartments and cytoplasmic behaviour**

*S. flexneri* and *L. monocytogenes* produce enzymes that partially degrade the phagocytic membrane, allowing the bacteria to enter the cytoplasm. Listeriolysin O, similar to the pore-forming streptolysin O, was the first virulence factor identified for *L. monocytogenes* (Gaillarid et al., 1986). However, in contrast to the other members of the hemolysin family, the enzymatic activity of listeriolysin O is highest at acidic pH as found in endosomal compartments and lower at neutral pH (Geoffroy et al., 1987), allowing the infected cells to remain viable once the bacteria replicate in the cytoplasm (Portnoy et al., 1992; Jones and Portnoy, 1994). In addition to listeriolysin O, the bacterium secretes two phospholipases, a phosphatidylinositol-specific phospholipase C encoded by *pclA* (Leimeister-Wacher et al., 1991; Mengaud et al., 1991) and a broad-range phospholipase C encoded by *pclB* (Narezque-Boland et al., 1992). Shigella also possesses a hemolytic activity enhanced at acidic pH (Clerc et al., 1986; Sansonetti et al., 1986). The product of the *ipab* gene has been shown to be involved in the disruption of
Fig. 1. Cytoskeletal rearrangements upon *Shigella flexneri* entry into HeLa cells. 

**A.** Transmission electron microscopy of *Shigella flexneri* strain SC301 invasion showing persistence and extension of cellular projections. 

**B** and **C**, hypothetical model of cytoskeletal rearrangements during (**B**) early entry stage and (**C**) later entry stage.  

- *a:* lateral zone of actin nucleation;  
- *b:* actin nucleation site under the bacterium;  
- *c:* subcortical actin filaments;  
- *d:* actin filaments nucleated by *b*.  

See text for details (reprinted from Adam et al., 1995, courtesy of P. Sansonetti, Institut Pasteur, Paris). Bar: 0.5 μm (x24,000).
phagocytic vacuoles (High et al., 1992). Its enzymatic activity is still unknown.

Once in the cytoplasm, neither Shigella nor Listeria appear to require specialized genes for survival. However, they undergo efficient motility processes to spread in and out of infected cells. Intracytoplasmic movements performed by Listeria and Shigella are due to the bacterially-induced formation of actin-rich comet tails (Fig. 2) (Theriot, 1995).

In Listeria, the product of the gene actA has been involved in the formation of the actin-rich comet tails (Las et al., 1995). ActA is a 639-amino-acid protein anchored in the bacterial membrane and it is asymmetrically distributed on the bacterial surface (Kocks et al., 1993). In infected cells, ActA co-localizes with the site of actin assembly and remains at the bacterial surface during bacterial movement (Kocks et al., 1993; Niebuhr et al., 1993). Expression of different parts of the actA gene demonstrated that the amino-terminus is necessary for actin nucleation (Friederic et al., 1995; Pistor et al., 1995). The rest of the molecule containing the polyproline region may be responsible for the actin dynamics associated with tail formation (Friederic et al., 1995) possibly by recruiting the profilin-binding protein VASP (Chakraborty et al., 1995).

In the case of S. flexneri, another gene involved in actin assembly has been identified. This is the icsA gene, whose product is a 120 kDa outer membrane protein (Bernardini et al., 1989; Lett et al., 1989; Goldberg et al., 1993). The N-terminal region of the protein contains several glycine-rich repeats, and the 479-amino-acid C-terminal region shares homology with ADA-I, the adhesin of diffusely adherent enteropathogenic E. coli (Benz and Schmidt, 1992). Purified IcsA displays weak ATPase activity (Goldberg et al., 1993). Cytoplasmic ligands of IcsA have not been identified. Like ActA, IcsA is also associated asymmetrically localized at the bacterial surface. These two proteins were shown to confer actin-comet-induced movement to the normally static Listeria innocua and E. coli (Kocks et al., 1995).

The actin-rich-comet movement is important not only to permit the bacteria to move in the cytoplasmic space, but also for the cell-to-cell spread. When the bacteria reach the edge of the cell, they push out membrane-bound protrusions (up to tens of micrometers in infected macrophages) to gain access into nearby cells, where they lyse these double-membrane compartments to reach the cytoplasmic space of the newly infected cell. Cadherin, a component of the intermediate junction, appears to be necessary for the proper structure and dynamics of the protrusions that bring bacteria inside adjacent cells (Sansonetti et al., 1994). In L. monocytogenes, the phospholipase PlcB is important for the lysis of the double bilayer (Vazquez-Boland et al., 1992). The phospholipase is secreted in inactivated form and activated by a secreted metalloprotease encoded by the mpl gene (Domann et al., 1991; Mengaud et al., 1991).

In Shigella, two genes are involved in cell-to-cell spread, the icsB gene (Allouis et al., 1992) and the vacJ gene (Fujita et al., 1994). However, the enzymatic activities of these two proteins are unknown.

Parasites occupying intracellular compartments

Mycobacterium

Early after infection, M. tuberculosis is found in a phagosome that contains MHC class I and class II molecules, as well as the early endosomal marker transferrin. MHC class I molecules are later cleared from the phagosome (Clements and Horwitz, 1995). Then, the bacteria modify both the protein composition of the phagosomes in which they reside and the interactions of these compartments with other host-cell organelles. M. tuberculosis is found in a phagosome.
Intracellular pathogens

tuberculosis arrests the maturation of the bacterial-containing phagosomes along the endosomal-lysosomal pathway, resides in a compartment with endosomal characteristics and avoids fusion with lysosomes (Armstrong and D'Arcy-Hart, 1971). Indeed, it is known that M. tuberculosis phagosomes privilege interaction with endosomes since fluid phase tracers internalized in endosomes can be found in bacteria-containing phagosomes (McDonough et al., 1993). Frehel and coworkers (1986) showed that M. avium phagosomes acquire horseradish peroxidase added to the culture medium after infection, but do not acquire lysosomal acid phosphatase, indicating a maturation arrest of M. avium phagosomes. Clements and Horwitz (1995) have found that M. tuberculosis phagosomes resist fusion with bovine-serum-albumine-gold-labeled secondary lysosomes. Even if late endosomal and lysosomal markers such as lysosomal membrane glycoproteins have been shown to be present in the phagosomes containing M. tuberculosis (Xu et al., 1994), their acquisition is strongly limited. Several mechanisms are proposed to explain the inhibition of the phagosome-lysosome fusion. They include the production of ammonia by different mycobacterial enzymes like arginase and urease (D’Arcy-Hart and Young, 1991) or the presence of special cell-wall glycolipids that could be inserted in the phagosome membrane to block the recognition by lysosomal docking proteins (Spargo et al., 1991). Another relevant feature of Mycobacterium phagosomes is the exclusion of the vacuolar proton pump (Sturilli-Koszycyki et al., 1994), the consequence of which is the inhibition of phagosome acidification-blocking the possible antibacterial action of many acid hydrolases.

Salmonella

In epithelial cells, S. typhimurium induces the formation of a spacious phagosome (Garcia-del Portillo and Finlay, 1994). It has been found that the early vacuole possesses MHC class I molecules, as well as lysosomal markers such as lysosomal glycoproteins (lgps) and lysosomal acid phosphatase (Garcia-del Portillo and Finlay, 1995a). However, the late endosomal marker mannose 6-phosphate receptor is not detected. Fluid phase markers rarely colocalize with intracellular bacteria (Finlay, 1994). Altogether, these data suggest that S. typhimurium enters into vacuoles which contain low levels of cell surface molecules, bypasses the late endosomal pathway and fuses with vesicles containing lgps that might arise directly from the trans-Golgi network (Garcia-del Portillo and Finlay, 1995b). Once the intracellular infection is established, Salmonella induces the formation of external filamentous structures rich in lgps that are connected to the bacteria-containing vacuoles (Garcia-del Portillo et al., 1993). The kinetics of formation of these lgp-rich structures closely parallel the kinetics of the parasite intracellular replication. Some avirulent mutants that are defective for intracellular replication fail to induce formation of these structures (Mills and Finlay, 1994). These observations seem to show that Salmonella-induced filaments containing lgps are linked to intracellular replication. However, the role of these filaments has to be defined. A novel gene called sifA, unique to Salmonella species, has been implicated in the formation of these filaments and in a virulence-associated intracellular phenotype of the bacteria (Stein et al., 1996).

Data are conflicting concerning the intracellular targeting and environment of S. typhimurium within macrophages. A previous report indicated that S. typhimurium resides within phagosomes that have fused with lysosomes (Carrol et al., 1979). Other works recently concluded that S. typhimurium inhibits phagosome-lysosome fusion and that dividing organisms are primarily found within unfused vesicles (Buchmeier and Heffron, 1991). In contrast to epithelial cells (see above), Alpuce-Aranda et al. (1994) reported that phagosomes containing bacteria are slowly acidified and that fluid phase markers fuse with internalized bacteria. Jones and Falckow (1996) showed that during the trafficking of S. typhimurium in cultured and bone-marrow-derived macrophages, the invading parasites almost immediately move into vacuoles enriched with the small GTPases rab7 and rab9, and that the ability of bacteria to reside within this intracellular niche is dependent upon the prompt acidification of the vacuoles. Several factors have been identified that confer enhanced survival during macrophage-specific infection. The best characterized is the PhoP/PhoQ system, a two component regulation system that activates at least five bacterial products (Miller, 1991), induced by low pH (Miller et al., 1992). One of the phenotypes that PhoP/PhoQ regulates, is to resist the action of bacterial cationic peptides which are involved in killing intracellular bacteria (Fields et al., 1989).

Brucella

B. abortus is a facultative intracellular bacterium that infects mainly macrophages and neutrophils, but whose interactions with non-phagocytic cells have also been characterized.

The early stages during intracellular infection are poorly understood. Gay et al. (1986) have presented evidence that during phagocytosis in mouse peritoneal macrophages, the phagocytes secrete fibronectin inside endocytic vacuoles, and a pseudocapsule is produced around endocytosed bacteria (Gay et al., 1981).

In neutrophils, B. abortus is phagocytozed without activation of the hexose monophosphate pathway and inhibits primary granule release (Kreutzer et al., 1979; Bertram et al., 1986). This latter activity is mediated by the release of 5'-guanosine monophosphate and adenosine, that inhibits the myeloperoxidase H2O2-halide antibacterial system (Canning et al., 1986). A cell wall component, the lipopolysaccharide (LPS) has also been implicated as a mediator of these effects in neutrophils.
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(Riley and Robertson, 1984) and has been proposed as a key molecule for the survival of the bacteria within the intracellular environment (Moreno, 1987). In fact, mutant bacteria that lack the most outer component of the LPS, the O-chain, do not survive inside cells as the native counterparts (Harmon et al., 1988). The Brucella LPS shows less biological activities than the LPS of enterobacteria, resulting in a poor activation of cells of the immune system (Rasool et al., 1992). However, transposon mutant strains of B. abortus show that in some cases the LPS-mutated bacteria can survive within macrophages as well as the non mutated Brucella, indicating that other molecules are also required to ensure the intracellular survival of the bacteria (Price et al., 1990).

Other activities observed after Brucella infection of macrophages include the possible inhibition of the phagosome-lysosome fusion by a soluble extract thought to contain sulfatides (Frenchick et al., 1985) without affecting phagosome-endosome fusion (Fig. 3; Pizarro-Cerdá et al., in preparation). The oxidative burst is not prevented by the bacteria (Bounous et al., 1993). The Brucella-containing phagosome has not been characterized yet. In non phagocytic cells like HeLa cells, the late endosomal and lysosomal markers mannose-6-phosphate receptor and lamp do not colocalize with B. suis after 24 or 48 hours of infection (O'Callaghan, personal communication), and the same pattern is found with B. abortus and B. melitensis in J774 macrophages (Pizarro-Cerdá, Desjardins, Moreno and Gorvel, unpublished results). In caprine trophoblasts as well as in African green monkey kidney fibroblasts (Vero cells), the bacterial replication seems to occur in the rough endoplasmic reticulum (Anderson and Cheville, 1986, Detilleux et al., 1990).

Presentation of intracellular-parasite antigens

Many intracellular parasites infect macrophages, perhaps taking advantage of the phagocytic activity of these cells to gain access into the intracellular space. However, macrophages are well known to play critical roles in the struggle against pathogens. These cells are not only involved in the initiation or maintenance of cell-mediated immune responses, but they also act as effector cells, functions that are linked to their ability to present endogenous and exogenous antigens, in the context of MHC class I and class II molecules (Umanue and Allen, 1987). On the other hand, the persistence of infections by intracellular pathogens raises the question about the strategies evolved by these parasites to escape or modulate the antigen presentation processes. The study of these processes has revealed new insights not only into the mechanisms used by intracellular pathogens to circumvent the host immune system, but also novel presentation mechanisms are being unraveled that feature the presence of non classical MHC molecules (Ojcius et al., 1996).

From the parasite side

Legionella pneumophila is a facultative intracellular

![Fig. 3. Fusion between endosomes and Brucella abortus-containing phagosomes in mouse peritoneal macrophages. Cells are infected overnight with B. abortus strain 19. During the last hour of infection, cells are incubated with serum albumin colloidal gold before sectioning and transmission electron microscopic observation. Arrow indicates fusion area. Bar: 0.25 μm (x130,000). (Pizarro-Cerdá et al., in preparation).](image-url)
bacterium that presents a direct approach to avoid contact between parasites antigens and MHC molecules: as the nascent phagosomal membrane appears during phagocytosis, selective incorporation of host plasma-membrane proteins occurs in the phagosomes, and remarkably MHC class I and class II molecules are excluded. The phagosomes later mature into a specialized compartment that is separated from the endocytic and exocytic routes (Clements and Horwitz, 1992, 1995).

In *Leishmania*-derived vacuoles, there are low levels of expression of MHC class I molecules. However, MHC class II molecules are heavily recruited during phagocytosis and the amount of vacuole-associated MHC class II proteins increases progressively after invasion (Lang et al., 1994). Further studies revealed that these MHC class II molecules are newly synthesized and still complexed with intact invariant chain that is rapidly degraded by proteases of macrophage origin, whereas the MHC class II molecules are internalized by the parasites and degraded by proteases of parasitic origin (De Souza-Leao et al., 1995).

In the case of *Mycobacterium* phagosomes, both MHC class I and class II molecules are present on the membrane of this compartment (Clements and Horwitz, 1995). The failure of acidification of this phagosome can account as a way of protection by the bacteria to avoid the degradation of parasitic peptides that could be loaded onto MHC molecules.

**From the host-cell side**

The first molecular indication that non classical MHC molecules are specialized for the presentation of bacterial antigens came from studies of the murine non classical MHC molecule H2 M3, which has an overall structure similar to the MHC class I molecules (Wang et al., 1995) but that binds preferentially peptides that contain a formylated amino terminus (Pamer et al., 1993). Since only prokaryotes (as well as chloroplasts and mitochondria) initiate protein synthesis with N-formylated methionines, the selectivity of H2 M3 makes it suitable for presenting microbial antigens.

Other molecules recently involved in the presentation of microorganism antigens are CD1a-e, molecules encoded by genes outside the MHC locus. Two mycobacterial antigens have been shown to be presented by the CD1b isoform: the mycolic acid, a class of long-chain fatty acids (Beckman et al., 1994) and LAM, a cell-wall component (Stieling et al., 1995). As LAM-specific T cells do not recognize mycolic acid presented by CD1b and vice versa, T cell recognition probably involves both the fatty acid and glycan moieties of LAM and mycolic acid, while lipid portions of these molecules are presumed to be associated with CD1b (Ojcius et al., 1996). Although it still remains to be proven that lipids can bind directly to CD1, it may be possible for lipids to lodge in the peptide-biding domain of CD1 because of its high content of hydrophobic residues (Calabi et al., 1989). Data from our laboratory also suggest that the Brucella LPS can bind CD1b and can associate with MHC class II molecules in a haplotype- and species-independent manner (Escola et al., 1994; Forestier et al., submitted).

**Perspectives: design of new vaccines**

Microbial pathogenesis has developed as a new discipline requiring a multidisciplinary approach that is based on classical microbiology, immunology, molecular genetics and cell biology. The new technologies within these fields of research have allowed investigators not only to better understand the molecular machineries used by pathogens to reach their intracellular targets, but also to modify this machinery in order to transform microbes in molecular tools for the development of new strategies of vaccination.

One original approach has been the use of attenuated intracellular parasites as antigen-delivery systems, in order to develop recombinant vaccine vehicles, which allow simultaneous expression of multiple protective epitopes of different pathogens (bacteria, viruses, protozoans) by the intracellular attenuated pathogen (Stover et al., 1991). Initial studies have focused in the use on *Salmonella* and *Mycobacterium* as delivery vehicles (Flynn et al., 1990; Aldovini and Young, 1991). However, the confinement of these bacteria in vacuoles restricted the accessibility of expressed foreign proteins to the host cell MHC molecules. Therefore, protection against heterologous pathogens has not been reported. Recently, *L. monocytogenes* has been used as a new model. *Listeria* offers the advantage of entering the cytoplasm of infected cells, allowing foreign proteins secreted by the bacteria to directly access the MHC class I antigen-processing pathway (Shen et al., 1995).

Another approach to design multicomponent antibacterial vaccines is the use of molecules like mycolic acid or LAM as immunizing antigens. As these molecules are only produced by mycobacteria, and not by eukaryotes, and as these ligands are presented by a small number of oligomorphic MHC-like molecules that are present throughout the human population, their potential use is relevant (Ojcius et al., 1996).

Finally, the ability of microbial molecules like the LPS of *B. abortus* to act as a carrier in generating humoral immune responses suggests that these molecules can be substituted for whole organisms in vaccine development (Bettas et al., 1993).

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