Salmonella typhimurium interations with host cells S4-1

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Abstrak

S. typhimurium mampu menginfeksi sel fagosit dan sel non-fagosit, dan menjadi patogen intrasel. Saat invasi sel epitel, S. typhimurium menyebabkan membran berkerut dan perubahan susunan aktin. Ia lalu menuju ke vakuola tertentu di mana ia bereplikasi. Yang berhubungan dengan replikasi adalah induksi suatu struktur unik hospes yang berbentuk filamen. Isolasi vakuola berisi S. typhimurium memungkinkan kami mempelajari protein mana dari hospes yang berhubungan dengan membran vakuola. Untuk memeriksa kejadian yang berhubungan dengan infeksi S.typhimurium pada mencit Balb/C dilakukan pemeriksaan hati yang terinfeksi menggunakan mikroskop konfokal. Inflamasi, lokasi bakteria, dan interaksi bakteri dengan sel hospes dianalisa dengan model infeksi salmonella dosis rendah ini. Teknologi ini akan memungkinkan kita untuk mempelajari peranan faktor virulensi dan pengaruhnyanya pada hospes selama infeksi menggunakan model yang yang relevan.

Abstract

S. typhimurium is capable of entering into phagocytic and nonphagocytic cells and functioning as an intracellular pathogen. During invasion of epithelial cells, S. typhimurium causes membrane ruffling and actin rearrangement as it enters. It then targets to a specialized vacuole where it replicates. Associated with replication is the induction of a unique host filamentous structure. Isolation of S. typhimurium containing vacuoles has enabled us to address which host proteins are associated with the vavuolar membrane. Confocal microscopy of infected livers was used to examine the events associated with S. typhimurium infections in Balb/C mice. Inflamation, the location of the bacteria, and bacterial interactions with host cells were examined in this low dose Salmonella infection model. This technology will allow us to address the role of individual virulence factors and their impact on the host during infection using a relevant model.

Introduction

Salmonellosis (diseases caused by *Salmonella* species) have several manifestations, ranging from gastroenteritis (food poisoning) to enteric fever and bacteremia. *Salmonella* species (especially *Salmonella typhimurium*) also represent resourceful microorganisms that can be used to investigate the complex interplay between a pathogen and its host. The ease with which *S. typhimurium* can be cultivated and genetically manipulated, in combination with the availability of tissue culture models and animal models, makes *S. typhimurium* a suitable organism for such studies. In this review, we focus on the intracellular stage of the *Salmonella* infection, describing the many complex cellular events which the bacteria inflicts upon the host cell, resulting in proliferation and ultimately infection and disease.

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Salmonella interaction with the intestinal epithelium

Pathogens transmitted by the oral route must cross the intestinal epithelium prior to the induction of a systemic (invasive) disease. The intestinal epithelium is composed predominantly of epithelial cells linked to each other by tight junctions. This arrangement provides an effective barrier to potentially harmful microorganisms present in the gastrointestinal lumen. *Salmonella* species, as well as several other pathogens, have the capacity to penetrate this barrier to cause infection in the host. The portal of invasion is not yet established since all epithelial cell types, although an increasing amount of data indicates that the initial sites of Salmonella infection are the Peyer's patches located in the ileum of the small intestine. The follicle-associated epithelium (FAE) overlaying these lymphoid tissues is characterized by the presence of M (microfold or membraneous) cells, specialized epithelial cells. Two groups have presented data from mouse ligated intestinal loop experiments strongly suggesting that M cells are the site of Salmonella entry into the host[1,2]. These data suggest

that even though Salmonella is able to invade any intestinal epithelial cell type[3], its major point of entry is the M cells within Peyer's patches.

Interactions of *S. typhimurium* with non-phagocytic cells

In this review our focus is on the intracellullar phase of the *Salmonella* infection. Therefore we refer readers to another review which covers *Salmonella* invasion in greater detail[4].

Salmonella invasion

The morphological changes of the ileal intestinal epithelial cells accompanying invasion of *S. typhimurium* in guinea pigs was described in detail in 1967[3]. Organisms in close contact with the epithelial cell surface induced degeneration of enterocyte microvilli. At later stages of the entry process, cytoplasmic projections from the host cells surrounded invading bacteria until they were contained within membrane-bound vacuoles. Over time, the apical surface of the invaded enterocyte regenerated and the cell apparently returned to normal. The use of cultured cell lines as experimental model systems has allowed insight at the molecular level into the bacterial adherence-invasion process described by Takeuchi. The adhesion to the epithelial cell surface is a transient state followed by either dissociation or immediate internalization. A massive cytoskeletal rearrangement is directly correlated to *Salmonella* entry into epithelial cells[5]. The *Salmonella*-induced ruffling of the host membrane is localized to the site of bacterial-host cell interaction and consists of rearrangement of host actin filament as well as other cytoskeletal proteins including talin, ezrin, -actinin, tropomyosin, and tubulin. Coincident with membrane ruffling, macropinosome formation and selective redistribution and internalization of host membrane proteins occurs[6,7]. Uptake of extracellular fluid was shown to specifically require signals related to bacterial invasion, since mutants unable to invade, but still adherent, failed to induce the formation of macropinosomes. The whole internalization process occurs within minutes and when completed, the bacteria resides within membrane bound vacuoles, and the cytoskeleton returns to its normal distribution.

Bacterial factors involved in *Salmonella* invasion

Entry into non-phagocytic tissue culture cells by *Salmonella* species appears to require several chromosomal loci, most of which are clustered between 58 and 60 minutes on the chromosome (reviewed in 4). The best characterize invasion locus, *inv*, is located at 59 minutes. The *invABC* and *invD* genes were originally isolated by their ability to complement a tissue culture invasion defect of *S. typhimurium*. Several other *inv* genes have since then been isolated and characterized. Another set of genes referred to as *spa* has been identified which are required for *Salmonella* entry into host cells[8]. The *spa* gene cluster overlaps to some extent the *inv* locus, forming contiguous operons. Many of the proteins encoded by the *inv/spa* gene cluster are homologous to proteins in other pathogenic bacteria which are implicated in the export of virulence determinants across the outer membrane via a type III secretion system[9,10]. Thus the *inv/spa* proteins in *Salmonella* constitute a type III secretion system invasion proteins. Type III secretion systems differ from other export system (type I and type II) in that protein export occurs via a *sec*-independent mechanism without processing of the amino terminal end of the secreted proteins.

Several proteins encoded in the *inv/spa* loci have been identified whose secretion is dependent on the type III secretion systems. Some of these (SspA, SspB or SipB, SspC or SipC, and SspD) exhibit significant homology to the invasion protein antigens (IpaA, IpaB, IpaC, and Ipa D respectively) of *Shigella* which also are secreted by a homologous type III export pathway encoded by the *mxiA/spa* loci. Not only are the protein sequence homologies between *inv/spa* products in *S. typhimurium* and *mxiA/spa* protein in *Shigella flexneri* remarkably conserved, but there is also a functional conservation between the homologous genes in the two species and mutations in *S. typhimurium* genes can be complemented by the cognate *S. flexneri* genes[8]. The exact role of Ssp (Sip) and Ipa proteins in mediating epithelial cell invasion is not clear, although they are critical for invasion. These secreted proteins might act as soluble components or as part of a filamentous surface appendage (invasome) transiently present on invasive *S. typhimurium* upon contact with epithelial cells[11].

Host factors involved in *Salmonella* invasion

The massive rearrangement of host cytoskeleton upon *Salmonella* entry as well as the requirement of host cell metabolism and energy for the uptake of *S. typhimurium* into non-phagocytic cells indicates that several host factors are involved in the internalization event. The bacteria probably transmit a signal which activates specific signal transduction mechanisms in the host cell resulting in the induction of the cy-

toskeletal rearrangements[12]. Activation of the host's phospholipase C upon bacterial contact produces two second messengers which further initiates complex signaling cascades. As a consequence, the host cell's [Ca^{2+}] is mobilized in a way which could then trigger cytoskeletal rearrangement and *S. typhimurium* internalization.

Upon infection, non-typhoidal *Salmonella* serotypes such as *S. typhimurium* provoke a large migration of neutrophils across the epithelial lining of the intestine, and an intestinal inflammatory response which leads to epithelial dysfunction[3]. This morphohological observation has now been confirmed in vitro and shown to occur due to the adhesion of *S. typhimurium* to the epithelial apical membrane[13]. However, the nature of the bacterial signal remains unknown.

Another effect of *S. typhimurium*-host cell interaction is stimulation of intestinal epithelial cells to produce pro-inflammatory cytokines[14]. Interleukin-8 release might play an important role in attracting neutrophils to the site of infection. This link between bacterial infection and the mucosal cytokine network further emphasizes the complexity of cell signaling events that occurs following bacterial infection.

Salmonella interactions with phagocytic cells

Salmonella are rapidly taken up by macrophages underlying the intestinal mucosa. It is not clear whether bacteria are internalized via macrophage phagocytosis, bacterium-mediated cell invasion, or a mixture of these two mechanisms. However, *Salmonella* entry into cultured macrophages is accompanied by membrane ruffling and macropinocytosis as is the case for epithelial cells[15]. Contrary to what is seen in invasion of epithelial cells, membrane ruffling occurs over a large portion of the cell surface of the macrophage often in areas without any apparent bacterial adhesion. This observation raises the possibility that either *Salmonella* or the infected macrophage produce a soluble factor which stimulates membrane ruffling. Other data suggest that bacteria-encoded invasins markedly enhance macrophage entry.

Recently, it has been shown that *S. typhimurium* can trigger programmed cell death (apoptosis) in cultured macrophages[16, 17]. As dicussed below, this event also appears to occur in vivo[18], although the significance of this event remains to be determined. Presumably the ability to kill host cells without stimulating an immune response (ie. via apoptosis) gives the pathogen an advantage.

The intracellular fate of *S. Typhimurium* in non-phagocytic cells

Upon invasion of both non-phagocytic and phagocytic cells, Salmonella is enclosed in membrane-bound vacuoles during their entire intracellular stage. Initially the bacteria are internalized individually in the vacuoles, but at later stages the vacuoles often fuse with each other. Following a lag period of approximately four hours, *Salmonella* begin to replicate intracellularly and the cells contain large vacuoles filled with bacteria 6-24 hours later, depending on epithelial monolayer type. Ultimately the cells presumably burst and release the organisms. Intracellular replication is an essential feature in *Salmonella* pathogenesis, since all replication-deficient mutants are highly attenuated for virulence in mice[19].

The *Salmonella*-containing Vacuole (SCV)

Once Salmonella has entered the host cell, the bacteria need to circumvent the host's antimicrobial killing mechanism to survive and to successfully develop a systemic infection. Several different strategies have evolved among the many pathogens which reside within vacuoles. To dissect the mechanism used by *Salmonella*, the composition of the *Salmonella*-containing vacuole (SCV), the intracellular environment the bacteria faces as well as the intracellular trafficking of the SCV have been examined.

Biogenesis of the SCV

During *S. typhimurium* invasion of non-phagocytic cells, selective aggregation and capping of several host plasma membrane proteins occur. These capped proteins colocalize with *S. typhimurium*-induced membrane ruffles. A preferential sorting mechanism appears to occur at the host's plasma membrane which ultimately defines the composition of the vacuolar membrane that encloses the bacterium which might be a mechanism for *Salmonella* to specify the formation of an intracellular niche which assures its survival and proliferation.

The intracellular environment

Little information exists about the microenvironment where intracellular pathogens like *Salmonella* reside and how intracellular bacteria within vacuoles obtain nutrients from the host cell. The use of a bacterial reporter gene system (lacZ) revealed that the concentrations of free Fe^{2+} and Mg^{2+} in the vacuole of epithelial cells are low, that the vacuole has mildly acidic pH and that lysine and oxygen is present[20].

The mechanism by which the infecting bacteria gains nutrients for replication is a key issue in pathogenesis. Some bacteria such as *Shigella* escape the membrane bound vacuole to get access to the nutrient rich cytoplasm of the host cell. However, *Salmonella* remain in vacuoles during the entire intracellular phase, and presumably, *Salmonella* has evolved other mechanisms of nutrient acquisition. Coincident with the start of *Salmonella's* intracellular replication, long and stable tubular-like structures appear which are connected the bacteria-containing vacuole[21]. The *salmonella* induced structures are present only in *Salmonella*-infected cells and can be visualized by the presence of lysosomal membrane glycoproteins (lgps) using immunofluorescence microscopy. Filament formation requires viable, intracellular bacteria and none of the prototrophic replication-deficient mutants mentioned above trigger their formation. Tubule formation requires a low intracellular pH, a functional protein synthesis machinery and at least one novel bacterial gene, *sifA*[22]. A deletion mutant of *sifA* is attenuated for virulence in mice, however, the precise function of the lgp-containing tubular structures is not clear.

Intracellular trafficking of the SCV

One of the host's defence mechanisms against foreign antigens (e.g. microorganisms) is to fuse the incoming antigen-containing endocytic vesicle with the terminal degradative compartment in the endocytic route, the lysosom. Lysosomes are a hostile microenvironment for bacteria. The development of mechanisms to escape the killing activity of the host cell's lysosomes is therefore of utmost importance for bacterial survival and proliferation. Mature lysosomes, which contain lysosomal membrane glycoproteins as their major membrane component, are formed in the late endosome compartment. Their formation leads to the subsequent degradation of the foreign antigen. However, recent investigations of the intracellular trafficking route used by *S. typhimurium* in epithelial cell suggest that the intracellular route of *Salmonella* corresponds to a unique pathway, different from the traditional phagocytic and endocytic routes[23]. This model postulates a trafficking route for lgp-containing vesicles which bypass the late endosome step and, instead, moves directly from the trans-Golgi network (TGN) to fuse with the *Salmonella*-containing vacuoles. It appears that the SCV fuses with a lgp-rich compartment different from mature lysosomes, and that trafficking of SCV bypasses the normal endocytic route having no interaction with the late endosomal compartment.

Normally, host vacuoles are not permissible for bacterial replication. However, data shows that SCV differs from the normal vacuolar compartments, and the unique features of the SCV provides an intracellular location for *Salmonella* to survive and proliferate within. This work suggests that *S. typhimurium* specifies of the SCV as a mechanism for intracellular survival. *S. typhimurium*-induced macropinosome-formation and sorting of host cell membrane proteins during bacterial invasion might be two ways to sequester components of the normal endocytic route of the host cell to promote *Salmonella*-specific trafficking of the SCV.

The intracellular environment of phagocytic cells

Professional phagocytic cells such as neutrophils and macrophages have a large repertoire of antimicrobial killing activities, including acidification of the bacteria-containing phagosome and degradation of its contents by antimicrobial proteins and peptides. These antimicrobial substances are released from lysosomes upon fusion with the phagosomes, and they include hydrolytic enzymes, defensins and enzymes that create reactive forms of oxygen and nitrogen. Several of the facultative intracellular pathogens avoid killing by phagocytic cells and persist in the host. Survival mechanisms used by intracellular pathogens include inhibition of phagosome acidification, inhibition of the respiratory burst, inhibition of the phagosome-lysosome fusion event, or escape from the phagolysosome to the host cells cytoplasm.

Characterization of the *Salmonella*-containing phagosomes including intracellular trafficking has recently gained support since studies of the pathophysiology of human and mouse typhoid fever suggest that macrophage survival is essential to *Salmonella* pathogenesis[24]. In the following sections we will discuss possible mechanisms for Salmonella survival within phagocytic cells.

Characterization of the *Salmonella*-containing phagosome

Once *Salmonella* has entered the macrophages, the bacteria persist within membrane bound phagosomes. However, *Salmonella*-containing phagosomes differ in size from the close-fitting phagosomes involved in conventional receptor-mediated phagocytosis being much larger, and are therefore called spacious phagosomes (SP)[25]. Formation of SP correlates with the ability of *Salmonella* to cause infection in hosts, and also requires the presence of one or more host factors. Other features specific for *Salmonella*-con-

taining murine phagosomes are their delayed and attenuated acidification. It takes 4-5 hours to reach pH<5.0 in phagosomes containing *S. typhimurium*, while phagosomes containing killed bacteria are rapidly acidified (pH<4.5 within 1 hour)[25]. These data suggest that viable bacteria are needed for inhibition of phagosome acidification or alternatively, viable bacteria are needed to signal a bacteria-specific uptake mechanism which ultimately delivers the bacteria to an intracellular location which is acidified slower than phagosome in the normal phagosome pathway.

Does *Salmonella* inhibit phagosome-lysosome fusion?

Conflicting data exists whether *Salmonella* inhibits phagosome-lysosome fusion as a mechanism of intracellular survival. A modified vacuole (SCV) has been described as the intracellular compartment in which *S. typhimurium* resides in epithelial cell[23]. In contrast, another report concluded that *S. typhimurium* resides within a fused phagolysosome, yet is able to attenuate endosome acidification[25], Their hypothesis was based on the colocalization of fluid endocytic tracers with the bacteria-containing phagosome, an observation which is in contrast to that seen in epithelial cells. Another possible conclusion is that the bacteria-containing vesicle is modified over so that it contains some of the lysosomal markers but not others, thereby specifying a unique phagolysosome different from the classical one. A previous report supports the view that *S. typhimurium* resides within phagolysosomes, and suggests that the bacteria have the capacity to resist destruction by lysosomal enzymatic activities as a mechanism of survival. However, this hypothesis is based solely on morphological data obtained by electron microscopy in which a modified lysosome would be difficult to detect.

Other results conclude that *S. typhimurium* inhibits phagosome-lysosome fusion within several types of macrophages[26]. The mechanism responsible for *Salmonella* inhibition of phagosome-lysosome fusion is unknown, but was shown to require viable bacteria which suggest that inhibition is a result of an active bacterial process. The contradictory data might be explained by the observation that two pools of intracellular bacteria have been reported, one which is static (possiby in a phagosome) and one which is fast growing (possibly avoiding lysosome fusion). Depending on the experiment performed, the influence of the two pools of bacteria within the cell may very which could lead to the discrepancy observed. Moreover, the intracellular survival of *Salmonella* has been shown to differ greatly between macrophages of different origins which also might influence the results reported.

Bacterial factors involved in intracellular survival

Survival of bacteria in the hostile environment of a macrophage requires differential expression of genes necessary for adaptation to stresses induced by lowered pH, differences in nutrient accessibility, and changes in osmolarity. Obviously, genes involved in the defense against macrophage killing mechanisms need to be expressed. Indeed, the bacterial protein shows drastic changes when the bacteria are grown within macrophages compared to those grown extracellularly. Two reports show that 30-40 proteins are induced while approximately 100 genes are repressed during intracellular growth[27,28]. Several stress-induced genes, including the heat shock proteins DnaK and GroEL, were detected among the intracellular induced genes. Other bacterial genes shown to be important in macrophage survival are *recA* and *recBC* suggesting that a functional DNA repair system is essential for *Salmonella* survival within phagocytes and for full virulence in mice.

In a large scale screen for bacterial factors that enchance the intracellular survival of *S. typhimurium*, more than 80 mutants were identified which showed a diminished capacity to survive within murine macrophages[24]. All mutants were avirulent in vivo suggesting that intracellular survival within macrophages is an essential feature for *Salmonella* pathogenesis. When a subset of the mutants were tested for replication in epithelial cells, they all replicated, indicating that the disrupted survival factors were macrophage specific. Further characterization of these mutants identified both genuine virulence genes, like the *phoPQ* genes as well as genes encoding housekeeping-or unknown functions.

The PhoP/PhoQ system is a two-component regulatory signal-transduction system which controls expression of a number of genes important for survival in macrophages and for virulence in mice[29]. Upon bacterial invasion the macrophage vacuolar environment is specifically detected by the sensor-kinase PhoQ. The ensuing phosphorylation of PhoP then leads to the expression of *pag* (PhoP-activated genes) genes. Maximum expression of *pag* genes coincides with the acidification of the *Salmonella*-containing vacuoles 3-6 hour after *Salmonella* entry into macro-

phages. However, the *PhoP/PhoQ* system also controls more than a dozen genes referred to as *prg* (PhoP-repressed genes) which are repressed by PhoP/PhoQ. Interestingly, both *pag* and *prg* genes are essential for virulence since a *phoP* mutation (PhoP^{C}) which results in constitutive *pag* transcription (and repression of *prg* genes) was attenuated for survival in macrophages and mouse virulence. Intracellular survival of *Salmonella* requires adaptation to a succession of environments, and it has been proposed that the proteins encoded by the *prg* genes may allow invasion and survival in the early phagosome whereas those encoded by the *pag* genes are required at later times during infection. The PhoP^{c} mutant has been used extensively to investigate the role of *prg* gene products in virulence. The vast array of phenotypes observed suggest that *prg* gene products are likely to be important in several events including induction of generalized membrane ruffling, macropinocytosis and the formation of spacious phagosomes during *Salmonella* internalization into macrophages, resistance to host antimicrobial peptides protein secretion involved in invasion of *S. typhimurium* into epithelial cells and induction of neutrophil transmigration across polarized epithelial cell monolayers.

The *phoP* locus has recently been demonstrated to play a crucial role in the inhibition of specific immunity[30]. Antigens of *S. typhimurium* were presented much more efficiently in a *phoP*$^{-}$ background compared to wild type or when the expression of *phoP* was constitutive (PhoP^{C}). The connection between the expression level of *phoP* and the processing and presentation of *Salmonella* antigens is not obvious. However, while wild type *Salmonella* enters macrophages in spacious phagosomes, it was shown that a PhoPc mutant entered macrophages in close-fitting phagosomes consistent with that expected for conventional receptor-mediated phagocytosis. Moreover, a Pho^{C} mutant of *Salmonella* is attenuated for virulence both in vivo and in macrophages. It is posible that the effect of PhoP on antigen processing and presentation is an indirect effect dependent on the bacterial route of entry into macrophages.

The genes discussed thus far are all located on the *Salmonella* chromosome. However, other genes necessary for virulence are located on the virulen plasmid in *S. typhimurium.* The exact role of the plasmid-encoded proteins in virulence is not clear but it does seem to allow for an increased growth rate of the bacteria in liver and spleen[31]. The five known virulence genes located on the plasmid (*spvABCD, spvR*) are regulated by the alternate sigma factor KatF[32]. KatF regulates genes that are induced during starvation conditions and during the stationary phase of bacterial growth and it was suggested that expression of the *spv* genes are required for prolonged survival within phagocytic cells.

Salmonella in vivo

In murine salmonellosis, these pathogens disseminate from the primary site of infection to organs of the reticuloendothelial system (RES) where they proliferate readily. The events needed for a successful *Salmonella* infection were poorly defined until a recent study examined *S. typhimurium* infections in mice using confocal microscopy[18].

Sites for *Salmonella* proliferation in vivo

To examine events that occur in vivo during Salmonella infection, two major experimental procedures have been used throughout the year : electron microscopical methods and quantitation of bacteria in homogenized organs. However, both methods have drawbacks. Total bacterial counts in the liver and spleen is a gross measurement that does not provide cellular detail. There are at least three different cell types in the uninfected liver (hepatocytes, Kupffer cells or resident macrophages, and endothelial cells) and additional cell types infiltrate the tissue due to the inflammatory response in infected organs. Consequently, homogenization of the entire liver does not reveal where and how the bacteria multiply. Furthermore, infection kinetic data obtained at the organ level might not reflect events at the cellular level. In contrast, electron microscopy examination of thin liver section gives us extremely detailed information. The limitation of this methods is that it requires very large infective doses of bacteria since it is only possible to study minute areas of the liver. This in turn might generate data that are not repesentative of entire organs. Based on electron of the microscopical studies, it was concluded that *Salmonella* multiply mostly extracellularly in the sinusoids of the liver and sometimes within the hepatocytes, and therefore *Salmonella* should not be considered as an intracellular pathogen[33]. However, very large inoculums were used in these experiments, as exemplified by intraperitoneal administration of 200.000 x 50% lethal dose of a virulent strain of *S. typhimurium* to highly susceptible mice. The question is how relevant this experimental design is to naturally acquired systemic *Salmonella* infections, which begin with the internalization of a few bacteria into the tissues or the sys-

temic circulation. The rapid infection induced by massive doses might instead trigger septic shock in the mouse due to the release of LPS (lipopolysaccharide) and toxic cell wall components into the circulation. Overwhelming doses of bacteria might also lead to different subpopulations of bacteria in the mouse. If only a fraction of the bacteria are phagocytosed by the macrophages, the remainder of the inoculum could quickly replicate and dominate since most bacteria are known to multiply faster extracellularly than intracellularly.

To examine *Salmonella typhimurium* interactions in mice using a more realistic infectious dose, Richter-Dahlfors et al.[18] infected mice with less than 100 bacteria i.v. Infected livers were harvested, fixed, and confocal microscopy used to identify single bacteria along with their corresponding host cell. Using this method, they were able to show that bacterial appearance in the liver coincided with the infiltration of neutrophils in inflammatory foci. At later stages of disease, the bacteria colocalized with macrophages, but not hepatocytes. They also found that bacteria were cytotoxic for phagocytic cells, and apoptotic nuclei were detected in the foci of infection.

It is a common view that *S. typhimurium* is able to survive intracellularly in macrophages as an important event during pathogenesis. In the study by Richter-Dahlfors et al.[18], they used confocal microscopy to demonstrate that all the detectable *S. typhimurium* in an infected liver are intracellular, inside macrophages or PMNs. This is consistent with the reports that the ability of *Salmonella* to survive or grow intracellularly varies in different macrophages. The importance of macrophages as the site for intracellular survival of *Salmonella* is further supported by the isolation of mutants unable to survive in macrophages in vivo[24].

Is *Salmonella* an intracellular pathogen?

Although *Salmonella* is generally considered to be a facultative intracellular pathogen, an alternative view questioning this notion has been proposed[33]. This hypothesis is based on experiments in which the animals has been challenged with very large does of virulent *S. typhimurium*. Under such condition the bacteria might avoid the intracellular stage of the infectious process, instead causing septic shock. Furthermore, the number of *Salmonella* to which the cell is initially exposed may determine whether the pathogen destroys the macrophages or the reverse is true. Mutants unable to replicate within epithelial cells in vitro as well as mutants defective for survival in macrophages in vitro are both avirulent in the mouse model. This suggests that the capacity to survive within host cells is an essential feature for *S. typhimurium* virulence. Therefore, is seems correct to classify *S. typhimurium* as an facultative intracellular pathogen.

Discussion

Salmonella pathogenesis is an example of complex interplay between bacteria and host cells. At all stages of the infection, *Salmonella* is capable of exploiting the preexisting host cell mechanisms in a variety of ways : I) invasion of bacteria is mediated via a sequence of events including cytoskeletal rearrangements and membrane ruffling, all caused by the bacterial subversion of the host's signal transduction pathways, II) the bacterium is able to direct the membrane composition of the vacuole it is located in within epithelial cells, thus specifying its own intracellular compartment, III) intracellular targeting of the *Salmonella*-containing vacuole (SCV) differs from the normal endocytic pathway, suggesting that *Salmonella* specifies its own trafficking route, IV) coincident with the onset of the intracellular replication of the bacteria, novel structures in the host cell appears. All of these events are probably part of the successful intracellular survival strategie(s) used by *S. typhimurium*. By specifying its own internalization mechanism, *S. typhimurium* is located intracellularly in a compartment which appears incapable of antigen presentation. The intracellular targeting of the *Salmonella*-containing vacuole is unique and separated from the host cell's normal endocytic and phagocytic routes, which further enhances the possibilities of intracellular survival. The localization of bacteria within the cells of infected animals might also provide safe sites for the bacteria where they avoid antigen presentation.

Although tissue culture experiments are invaluable for the study of pathogenic features of the microorganisms and of the basic mechanisms in their interaction with the host, the histopathology of the cells and tissues affected by *Salmonella* needs to be considered to fully understand the pathogenesis of *Salmonella* infections. Data obtained from experiments using the mouse model of typhoid fever are often contradictory, and one has to be cautious in their interpretaion. One basic question to keep in mind is how relevant the experimental model is to events occuring in naturally acquired infections. Of all the mutants charac-

terized in tissue culture models, very few have been characterized at a cellular level in the mouse model, probably due to the limitation of experimental approaches in vivo. However, recently described approaches to investigate the role of the individual cell types in the organs using FAC sorting and cell purification are new and promising ways of ivestigating the host-pathogen interactions in the pathogenesis of systemic salmonellosis. Using these approaches to characterize the in vivo phenotypes of mutants whose phenotypes already are known in vitro will provide further insight into the complex mechanisms of *Salmonella* pathogenesis.

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References

1. Clark MA, Jepson MA, Simmons NL, Hirst BH. Prefential interaction of *Salmonella typhimurium* with mouse Peyer's patch M cells. Res Microbiol 1994; 145: 543-52.
2. Jones BD, Ghori N, Falkow S.*Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med 1994; 180:15-23.
3. Takeuchi A. Electron microscope studies of experimental *Salmonella* infection. I Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am J Pathol 1967; 50:109-36.
4. Galan JE. Molecular genetic bases of *Salmonella* entry into host cells. Mol Microbiol 1996; 20: 263-71.
5. Francis CL, Starbach MM, Falkow S. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol Microbiol 1992; 6: 3077-87.
6. Garcia-del Portillo F, Finalay BB. *Salmonella* invasion of nonphagocytic cells induces formation of macropinosomes in the host cell. Infect Immun 1994; 62: 4641-5.
7. Garcia-del Portillo F, Pucciarelli MG, Jefferies WA, Finlay BB. *Salmonella typhimurium* induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. J Cell Sci 1994; 107: 2005-20.
8. Groisman EA, Ochman H. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J 1993; 12: 3779-87.
9. Finlay BB, Falkow S. Common themes in microbial pathogenesis revisisted. Microbiol Mol Biol Rev1997; 61:136-69.
10. Finlay BB, Cossart P. Exploitation of mammalian host cell functions by bacterial pathogens. Science 1997; 276: 718-25.
11. Ginocchio CC, Olmsted SB, Wells CL, Galan JE. Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. Cell 1994; 76:717-24.
12. Rosenshine, I., Finlay, B.B. Exploitation of host signal transduction pathways and cytoskeletal functions by invasive bacteria. Bioessays 1993; 15:17-24.
13. McCormick BA, Miller SI, Carnes D, Madara JL. Transepithelial signaling to neutrophils by salmonellae: a novel virulence mechanism for gastroenteritis. Infect Immun 1995; 63: 2302-9.
14. Klimpel GR, Asuncion M, Haithcoat J, Neisel DW. Cholera toxin and *Salmonella typhimurium* induce different cytokine profiles in the gastrointestinal tract, Infect Immun 1995; 63: 1134-7.
15. Alpuche-Aranda CM, Racoosin EL, Swanson JA, Miller SI. *Salmonella* stimulate macrophage macropinocytosis and persit within spacious phagosomes. J Exp Med 1994; 179: 601-8.
16. Chen LM, Kaniga K, Galan JE. Salmonella species are cytotoxic for cultured macrophages. Mol Microbiol 1996; 21: 1101-15.
17. Lindgren SW, Stojiljkovic I, Heffron F. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. Proc Nat'1 Acad Sci USA 1996; 93: 4197-201.
18. Richter-Dahlfors A, Buchan AMJ, Finlay BB. Murine salmonellosis studied by confocal microscopy: *S. typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagotcytes in vivo. J Exp Med In press 1997.
19. Leung KY, Finlay BB. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. Proc Natl Acad Sci USA 1991; 88: 11470-4.
20. Garcia-del Portillo F, Foster JW, Maguire ME, Finlay BB. Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. Mol Microbiol 1992; 6: 3289-97.
21. Garciad-del Portillo F, Zwick MB, Leung KY, Finlay BB. *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoprotein in epithelial cells. Proc Natl Acad Sci USA 1993; 90: 10544-8.
22. Stein MA, Leung KY, Zick M, Garcia-del Portillo F, Finlay BB. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. Mol Microbiol In press.
23. Garcia-del Portillo F, Finlay BB. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypass compartments with mannose 6-phosphate receptors. J Cell Biol 1995; 129: 81-97.
24. Fields PI, Swanson RV, Haidaris CG, Heffron F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci USA 1986; 83: 5189-93.
25. Alpuche-Aranda CM, Swanson JA, Loomis WP, Miller SI. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc Natl Acad Sci USA 1992; 89: 10079-83.

26. Buchmeier NA, Heffron F. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect Immun 1991; 59: 2232-8.

27. Buchmeier NA, Heffron F. Induction of *Salmonella* stress protein upon infection of macrophages. Science 1990; 248: 730-2.

28. Abshire KZ, Neidhardt FC. Growth rate paradox of *Salmonella typhimurium* within host macrophages. J Bacteriol 1990; 175: 3744-8.

29. Miller SI. PhoP/PhoQ: macophage-specific modulators of *Salmonella* virulence? Mol Microbiol 1991; 5: 2073-8.

30. Wick M, Harding CV, Twesten NJ, Normark SJ, Preifer JD. The phoP locus influences processing and presentation of *Salmonella typhimurium* antigens by macrophages. Mol Microbiol 1995; 16: 465-76.

31. Gulig PA, Doyle TJ. The Salmonella *typhimurium virulence* plasmid increases the growth rate of *salmonellae* in mice. Infect Immun 1993; 61: 504-11.

32. Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, Guiney DG. The alternative sigma factor katF (*rpoS*) regulates *Salmonella* virulence. Proc Natl Acad Sci USA 1992; 89: 11978-82.

33. Hsu HS. Pathogenesis and immunity in murine salmonellosis. Microbiol Rev 1989; 53: 390-409.