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Review

# Host and bacterial factors in listeriosis pathogenesis

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## Abstract

Members of the Genus *Listeria* are ubiquitous environmental saprophytic microorganisms. If ingested they can cause a severe disseminated disease (listeriosis) that has a high mortality rate, the highest of any food-borne pathogen, even with antibiotic therapy. Central to the high mortality rate is the hallmark characteristic of the microorganism to grow intracellularly. The presence of listeriae in food processing plants has resulted in many outbreaks of human disease and large scale recalls of processed foods. Despite the ubiquity of the microorganism, the actual disease rate (those animals showing disease signs over those exposed) is quite low and disease is almost always associated with an underlying predisposition (pregnancy being the most common in otherwise normal individuals). There are many features of the pathogenesis of listeriosis that have remained mysterious despite the extensive use of the microorganism in the study of cell-mediated immunity and intracellular growth. Informational advances such as the sequence of the mouse and listerial genomes, and technical advances such as the discovery of listeria-susceptible mouse strains, may renew interest in the study of the natural pathogenesis of the disease. This may be further facilitated by studies that employ the natural inoculation route and mimic common predisposing conditions witnessed in victims of natural outbreaks.

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## 1. Introduction

It seems appropriate that the properties of the genus *Listeria* should occasionally be reviewed in a journal concerned with veterinary pathogens. After all, the microorganism, later to be named *Listeria monocytogenes*, was isolated from a sick rabbit (Murray et al., 1926) and it was recognized as a veterinary pathogen for at least 10 years before a connection to human disease was suspected (Burn, 1936). Also, the food-borne nature of listerial infections was familiar to veterinarians since the late 1930s (Olafson, 1940) a fact that has been documented in humans for less than 25 years (Schlech et al., 1983). In this short review, we concentrate on the molecular mechanisms of listerial pathogenesis that are ostensibly similar in all animals. Other excellent and recent reviews covering various aspects of listeriosis are available (Dussurget et al., 2004; Portnoy et al., 2002; Vazquez-Boland et al., 2001) as well as the standard, veterinary-focused review of Low and Donachie (1997). These reviews, and others (e.g., the half-century classics (Gray and Killinger, 1966; Seeliger, 1961)) were consulted frequently in the writing of this article.

## 2. The microorganism and its environment

There are seven species of *Listeria*, of which two are pathogenic (*L. monocytogenes* and *L. ivanovii*). The former causes disease in humans and other animals, the latter causes disease almost exclusively in ruminants. Consequently, the properties of *L. monocytogenes* are most generally applicable and we will limit ourselves to a discussion of this species. *L. monocytogenes* is a Gram-positive facultatively anaerobic bacterium that can be isolated from soil, animal feed, water, feces, and tissues from a variety of invertebrate and vertebrate animals, including man

(Cooper and Walker, 1998). It can be readily identified using standard biochemical tests as it grows well in the laboratory over a broad temperature range on a variety of carbon sources. There are a number of *L. monocytogenes* serovars that are based upon surface carbohydrate (O or somatic) and flagellar antigens and given alphanumeric designations (Farber and Peterkin, 1991). *L. monocytogenes* is a common contaminant of foodstuffs and has been recovered from raw vegetables, milk products, fish, poultry, and meats at rates of 15–70% (Farber and Peterkin, 1991). Food-borne outbreaks of listeriosis are relatively common (Morbidity and Mortality Weekly Report [MMWR] 2003, 52, 340) and when such outbreaks occur, the percent of fatalities can exceed that of other more notorious food-borne pathogens, such as *Clostridium botulinum* (Mead et al., 1999). Listeriae grow very well at reduced temperatures compared to other mesophilic medically important microorganisms. This characteristic has contributed both to their continued foodstuff ubiquity in the age of refrigeration and to the classical means of isolating them from clinical and environmental sources (Gray and Killinger, 1966). In 2002, a listeriosis outbreak resulted in seven deaths and the largest recall of any agricultural product in history (27.4 million pounds of processed meat [MMWR 2002, 51, 950]). These properties likely contributed to the placement of *L. monocytogenes* on the list of select agents ([http://www.niaid.nih.gov/biodefense/bandc\\_priority.htm](http://www.niaid.nih.gov/biodefense/bandc_priority.htm)).

Whereas there are several clinical manifestations of listeriosis, the common features of the disease are: (i) a contaminated food source and an oral entry route; (ii) colonization of the intestine; (iii) intestinal translocation; (iv) replication in the liver and spleen; (v) resolution (dependent upon T cell-mediated immunity) or hematogenous spread to other organs. In humans, stages ii and iv often produce symptoms (enteritis, or a febrile flu-like condition, respectively)

that will prompt the victim to seek medical attention (Salamina et al., 1996). In other animals, detection at these stages is less likely and signs of disease typically involve stage (v) sequelae. The most common of these sequelae are abortion in pregnant animals or, in non-pregnant animals, neurological signs reflective of encephalitis.

From an agricultural standpoint, the problem of listeriosis is most readily associated with food safety and public health (<http://www.foodsafety.gov/~dms/lmrisk.html>) rather than unacceptable losses from disease in agriculturally important animals (Kathariou, 2002). Whereas losses do occur, most outbreaks exhibit a well-defined profile that has varied little in the 60 years since listeriosis has been recognized as a disease (Low and Donachie, 1997). The typical scenario involves ruminants fed silage (Gray, 1960). In many cases the fermentation process that creates silage has not lowered the pH sufficiently to prevent overgrowth of the ubiquitous listeriae (Fenlon, 1985). Animals predisposed to infection via environmental or physiological factors (pregnancy is the most common normal physiological challenge (Siegmán-Igra et al., 2002)) are the typical victims. In humans, chronic illnesses, extremes of age, liver disease, malignancies, heart disease, and diabetes can also be involved (Goulet and Marchetti, 1996; Schuchat et al., 1992). In all animals the mortality rate can be quite high, even with appropriate and active antibiotic intervention (Hof et al., 1997). Indeed, the feature of listeriosis that is most unacceptable is not its incidence, but rather the high mortality rate in the face of active antibiotic therapy (Gellin and Broome, 1989).

Antibiotic therapy is often ineffective for several reasons. However, the most central reason may be the ability of the microorganism to multiply intracellularly and spread cell-to-cell without leaving the protective environment of the host's cells. This property not only limits the choice of antibiotic therapy, but also necessitates a vigorous cell-mediated host immune response. It is a testament to livestock breeders that this response is usually adequate to prevent clinical signs of infection: domestic animals do quite well in the presence of relatively high environmental levels of listeriae (Nightingale et al., 2004). Indeed, a high proportion (ca. 6%) of animals, including man, normally carry listeriae as part of their fecal flora without any obvious outward effects (Husu

et al., 1990; Schuchat et al., 1991). Also, healthy humans commonly possess sensitized T lymphocytes to listeriae (Munk and Kaufmann, 1988). The ubiquitous saprophytic nature of listeriae and the asymptomatic fecal carriage combine to make the possibility of eliminating listeriae unrealistic. Nevertheless, a better understanding of the features of listeriae that make them pathogenic may result in more refined ways of treating the disease and recognizing particularly pathogenic clones in the food chain (Evans et al., 2004; Jacquet et al., 2004; Nightingale et al., 2005). Since the listeriae are accidental pathogens and consequently have not evolved in association with a particular host (e.g., *Brucella abortus* and cattle) or hosts, the collection of genetic factors that produce an unusually pathogenic clone may be large and varied. Indeed, efforts to identify factors common to clinical isolates (as opposed to environmental isolates) using surrogate in vitro and in vivo tests have produced somewhat mixed results (Jacquet et al., 2004; Jaradat and Bhunia, 2003; Pine et al., 1991; Van Langendonck et al., 1998). Presently all *L. monocytogenes* isolates from food processing plants or in food stuffs are viewed as pathogens. Given that a virulent subpopulation of an innocuous environmental food contaminant may be selected in hosts following a massive inoculation, the current no-tolerance policy is probably prudent.

### 3. Host immune defenses

*L. monocytogenes* has been used for decades by immunologists to study features of cell-mediated immunity. It is somewhat ironic that these studies, while revolutionizing our understanding of the immune system, did not model the normal pathogenesis of listeriosis. That is, investigators initiated infections with parenteral rather than oral inoculations and most commonly examined the innate or adaptive ability of the immune system to limit the infection to the liver and spleen. Whereas more natural routes are being modeled increasingly often (Beretich et al., 1998; Czuprynski et al., 2003; Hamrick et al., 2003; Lecuit et al., 2001), most studies still employ parenteral inoculations. Nevertheless, the information accrued over decades using mice parenterally inoculated have produced a large body of clear and detailed information on the

host's immunological response to listeriae and by extension, other intracellular pathogens.

Host defense against *L. monocytogenes* involves non-specific innate immunity and specifically acquired (adaptive) T cell-mediated immunity (reviewed by Unanue (1997)). Innate immune responses are mounted almost immediately after the onset of infection and serve to control the acute phase of infection until a specially acquired T cell-mediated immune response is generated to eradicate pathogens residing intracellularly. After their lodgment in the liver and spleen, listeriae enter and multiply in both professional phagocytic cells (polymorphonuclear granulocytes [PMNs], macrophages, dendritic cells [DC]) as well as other cell types (enterocytes, hepatocytes). Control of listerial infection depends on the rapid activation of innate immune mechanisms chiefly through toll-like receptors (TLRs). TLRs are expressed on a number of different cells involved in innate immunity and recognize pathogen-associated molecular structures. Signals generated through TLRs mediate the induction of pro-inflammatory cytokines and other important immune mediators. TLR2 is particularly important in the protection against infections by Gram-positive bacteria, including *L. monocytogenes* (Takeuchi et al., 1999; Torres et al., 2004). TLR2 recognizes several bacterial-associated molecules, including lipoteichoic acid, lipoproteins, and peptidoglycan. TLR2 signaling triggers NF- $\kappa$ B pathway-dependent innate immune mechanisms including, the production of pro-inflammatory cytokines (e.g., tumor necrosis factor [TNF]) and chemokines that are essential for recruiting PMN and blood-borne monocytes to infectious foci early in infection (Havell, 1989). On the first day of infection, natural killer cell activity is elevated and these cytolytic cells produce interferon gamma (IFN- $\gamma$ ), which in turn, endows macrophages with an augmented capacity to produce cytokines (TNF and IFN- $\gamma$ ) and molecules (e.g., nitric oxide) that have pivotal roles in innate immunity (Bancroft et al., 1989; Dunn and North, 1991; Havell, 1993).

Macrophages and DC also function as antigen presenting cells in the generation of specific antilisterial T cell-mediated immunity. Following their uptake by these phagocytes, listeriae evade destruction in the hostile confines of the phagosome by secreting the virulence factor, listeriolysin-O (LLO) (Gedde

et al., 2000). This pore forming toxin enables listeriae to escape from the phagosome into the cytosol where they multiply and polymerize actin on their outer envelope. Actin polymerization results in the formation of actin tails that both propel the listeriae through the cytoplasm and facilitate the intracellular invasion of other host cells (reviewed by Cossart (2002)). In DC, listerial proteins (e.g., LLO) are processed and then displayed externally on the membranes in context with major histocompatibility complex (MHC) class I and MHC class II molecules to activate, respectively, naive CD8+ and CD4+ T cells. DC also produce costimulatory factors such as IL-12 which drive activated CD4+ T cells to become Th1 helper cells that secrete factors (e.g., IFN- $\gamma$ ) to promote CD8+ cytolytic T lymphocyte (CTL) responses.

MHC class I restricted CD8+ CTL mediate the resolution of a primary infection and are responsible for long term secondary (memory) antilisterial immunity (Ladel et al., 1994; Serbina and Pamer, 2003). Sensitized CD8+ CTL recognize and bind to LLO peptides presented in the groove of MHC class I molecules on the surface of infected cells. These activated CD8+ CTL destroy cells displaying LLO peptides complexed with MHC class I molecules by producing cytokines (IFN- $\gamma$  and TNF), perforin and granzymes (San Mateo et al., 2002; Wing and Gregory, 2000). IFN- $\gamma$  activates the listeriocidal activity of macrophages and modifies the phagosome in a manner that prevents the escape of listeriae from the hostile confines of the phagosome into the cytosol (McCaffrey et al., 2004). This cytolysis of infected host cells harboring listeriae make the formerly protected intracellular pathogens susceptible to destruction by activated macrophages. Peak numbers of activated CD8+ CTL are present in the host on or about day seven of listeriosis which corresponds to the time when the greatest level of T cell-mediated antilisterial immunity can be adoptively transferred to naive recipient mice (Havell et al., 1982). Studies have also established that during listeriosis, the composition of T cells populating the spleen and the intestinal mucosa differ (Huleatt et al., 2001). This finding and that of others (Pope et al., 2001; Rakhmievich, 1994) indicate distinct immune mechanisms are brought into play at different anatomical sites. These findings support the importance of a natural inoculation route in experimentally modeling listerial infections.

The results of studies by Busch et al. (1998), revealed that after day 7 of infection, the numbers of specifically activated CD8+ CTL effector cells contract as the antigenic load diminishes. CD8+ CTLs target DC expressing listerial antigens and prevent the further activation of naive T cells such that there will not be an over expansion of CD8+ CTLs (Wong and Pamer, 2003). Thereafter, long-lived memory immune CD8+ T cells survey tissues for reinfesting listeriae. The numbers of memory CD8+ T cells are regulated by CD4+CD25+ T cells to prevent over expansion of memory CD8+ CTL during anamnestic responses, which could result in excessive inflammation and tissue destruction (Kursar et al., 2002).

Antibodies (IgG and IgA) to listerial antigens are produced by the host, and until recently it was widely believed that antibodies were not protective based on the findings of Mackaness (1962). However, it has now been reported that specific anti-LLO antibodies protect the host from listerial infection (Edelson et al., 1999). Evidence indicates that LLO specific antibodies in endocytic vesicles of the macrophage encounter *Listeria*-containing vacuoles. LLO produced by the listeriae is then neutralized by the anti-LLO antibodies, and the listeriae are retained within the phagosome (Edelson and Unanue, 2001). The inability to escape from the phagosome would render

the phagocytosed listeriae susceptible to the killing mechanisms expressed in these vesicles.

#### 4. Dissemination in gravid and nongravid animals

Failure of the host to confine the microorganisms to the liver and spleen results in hematogenous spread with lodgment and expansion of the listeriae in other sites. In natural infections, a single factor determines the lodgment site: pregnancy (Fig. 1). Pregnancy greatly increases the risk of listeriosis in virtually all susceptible animal species (Gellin et al., 1991; Low and Donachie, 1997; Shinomiya et al., 1986). More than 40 species of wild and domestic animals are susceptible to listerial infection (Seeliger, 1961) and listeriosis is a significant concern in the production of caprine, ovine, and bovine species (Vet. Rec. 1983. 112, 314). Of the susceptible domestic animal species, the ones most clearly at risk are pregnant ruminants fed contaminated food — usually silage (Low and Donachie, 1997; Sanaa et al., 1993). These risk factors are strikingly similar to humans where the pregnant host consumes improperly prepared or stored food (Armstrong, 1995). In humans, approximately 33% of clinically documented cases of listeriosis are in pregnant women (Armstrong, 1995) and pregnant

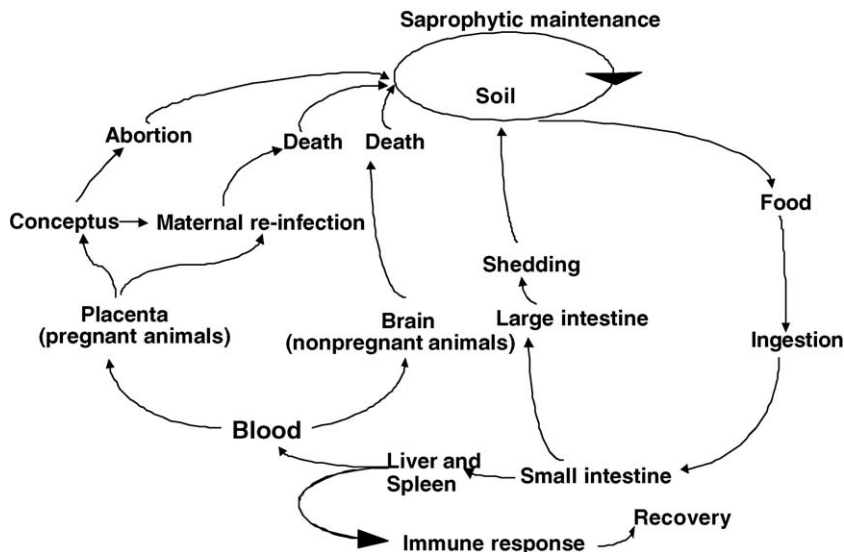


Fig. 1. Listerial saprophytic and infectious cycles illustrating the central branch point of blood-borne bacteria effected by pregnancy.

women constitute approximately 60% of all cases (male and female) aged 10–40 years (Ciesielski et al., 1988). In one outbreak, 65% of those contracting listeriosis were pregnant and 63% of those pregnancies ended in fetal or neonatal death (MMWR 1985. 34, 357–60).

Despite the well-known association of listeriosis with pregnancy, there is incomplete agreement on how pregnancy increases disease risk. Laboratory-based investigations have been complicated by the varying virulence of listerial strains (Barbour et al., 2001), the various routes of inoculation employed, and the normal resistance of the preferred host (the mouse) to infection via the natural (alimentary) route (Lecuit et al., 2001). Some reports suggest that the generalized immune impairment demonstrable in the gravid mouse is a factor in increased disease susceptibility (Abram and Doric, 1997; Nakane et al., 1985). However, the degree to which this impairment affects antilisterial capacity is disputable. There is more agreement that events at the fetal/maternal boundary are critical in the progress of the disease (Guleria and Pollard, 2000; Lu et al., 1989; Redline and Lu, 1987, 1988). This boundary is defined in all eutherian animals by the embryonic layer composing the trophoblast (Ramsey, 1982). The trophoblastic epithelium, and the fetal membranes derived from this stem cell, have long been regarded as instrumental in maintaining the integrity of the fetus not only from bacterial invasion but also from the maternal immune system that would normally view the fetus as a foreign body (Bainbridge, 2000).

The degree to which the trophoblastic layer presents a barrier to circulating bacteria is an understudied area. One reason that so little work has been done is that viviparous species have evolved drastically different forms of placentation (Mossman, 1987; Noden and deLahunta, 1985). Whereas these diverse anatomies are thought to owe their heterogeneity to evolutionarily ad hoc solutions to the central problem of fetal rejection (Medawar, 1953), from the perspective of an infectious agent, it means that the innate antimicrobial defense mechanisms of trophoblasts from one species may differ from those in another. Nevertheless, in all animals in which listeriosis has been reported, pregnancy stands out clearly as a predisposing factor. Recent work in mice, guinea pigs, and human cell culture has

produced provocative, if not entirely compatible, results on some of the listerial factors that are important for intrauterine infections in the gravid host (Bakardjiev et al., 2004; Hamrick et al., 2003; Lecuit et al., 2004).

The means by which the gravid uterus becomes infected with listeriae is also of great interest. While long assumed that uterine infection resulted from hematogenous spread, ascending routes have also been proposed (Gray and Killinger, 1966) especially in the case of neonatal listeriosis (Silver, 1998). Also, the question as to whether the bacteria have a tropism for the gravid uterus has produced some disagreement and (or) misunderstandings. There seems to be agreement that both the pregnant uterus and the central nervous system possess an environment favoring unrestricted growth of listeriae (Vazquez-Boland et al., 2001). However, there is little to suggest that the microorganisms lodge in these tissues due to some initial attraction for these sites (e.g., receptor–ligand interactions). For example, in animals where the gravid female typically shows no outward signs of infection (ruminants), healthy calves have been born to cows chronically shedding listeriae in their milk (Cooper and Walker, 1998) and between 1 and 12% of pregnant women have been reported to carry detectable fecal levels of listeriae asymptotically (Silver, 1998). Similarly, in some studies, a relatively small percentage (ca. 20%) of gravid women presenting with symptoms of listeriosis had fetal complications (Ciesielski et al., 1988) even though fetal complications likely contribute to disease severity and thus the likelihood of presenting (MacGowan et al., 1991). Our own studies in mice suggest that a relatively small proportion of pregnant animals with demonstrable listeriae in their livers and spleens actually acquire an intrauterine infection. However, extrauterine infections do produce lower birth weight pups — perhaps due to diffusible listerial factors that put stress on the developing embryo (Fig. 2). Whereas tropism for the uterus was initially indicated in guinea pigs (Bakardjiev et al., 2004) following parenteral inoculation, subsequent findings make this possibility less likely (Bakardjiev et al., 2005). Consequently, coincidental infection of the uterus followed by unrestricted growth may better explain most observations (Hamrick et al., 2003; Redline and Lu, 1987).



Fig. 2. Pericardial edema (arrow), a sign of fetal distress in an embryo at 9.5 gestational days and 2 days after maternal intragastric inoculation of *Listeria monocytogenes*.

Presently, case studies suggest the gravid host is most susceptible to listeriosis during the later stage of pregnancy. However, this conclusion is seldom adequately qualified — perhaps because, with isolated exceptions (Pezeshkian et al., 1984), disease presentation correlates well with a general depression in the host's cell-mediated immunity late in gestation (Bortolussi et al., 1984; Low and Donachie, 1997; Weinberg, 1984). It is rarely pointed out that miscarriages (i.e., abortions at the embryonic stage rather than at the later fetal stage) are typically not followed up microbiologically and occur at such a time that the gravid host may display fewer signs of infection or (in the case of humans) have those signs passed off as a normal part of pregnancy (e.g., morning sickness). Indeed, in production animals, miscarriages could be missed entirely and registered only as infertility. Also, in later pregnancy placental anemic infarcts become relatively common and may be an additional source of access to the fetus that has

nothing to do with a systemic depression in the gravid host's immune status (Gray and Killinger, 1966). Experimental studies in mice, where the timing of impregnation and inoculation can be tightly controlled, indicate that gravid females become prone to disease at early times in pregnancy (Abram and Doric, 1997; Hamada et al., 1981; Hamrick et al., 2003). Further, a number of reports indicate that there is little change in susceptibility of gravid mice to a disseminated infection at any time during gestation. Rather, the infection, once acquired, is more severe due (evidently) to infection of the intrauterine contents and subsequent unrestricted growth (Hamrick et al., 2003; Klink and Rudnicka, 1995; Lammerding et al., 1992; Redline and Lu, 1987).

In nongravid animals, the most common sequelae of listerial infection is invasion of the central nervous system (CNS). In humans, United States surveillance indicates that *L. monocytogenes* is the second leading cause of bacterial meningitis in patients younger than 1 month or older than 60 years (Schuchat et al., 1997). Meningitis and meningoencephalitis are the two most common CNS manifestations in humans (Bula et al., 1995; Goulet and Marchetti, 1996; McLauchlin, 1990; Mylonakis et al., 1998; Pollock et al., 1984). The majority of brain abscesses occur in individuals that are compromised by underlying medical conditions or which are receiving immunosuppressive therapy. In contrast, rhombencephalitis, a primary infection of the brain stem, is found predominantly in noncompromised adults. Meningitis and meningoencephalitis are present in ruminants (Low and Donachie, 1997) as well as brain stem encephalitis (Campero et al., 2002). It is likely that some underlying predisposed state may be normally required in ruminants for clinical disease presentation (Low and Donachie, 1997). If so, the factors producing that state are obscure: even the animals showing the highest natural incidence of listeriosis (sheep) are difficult to infect via the oral route experimentally (Low and Donachie, 1997).

The means by which listeriae gain access to nerve cells and cross the blood brain barrier may be varied and has been the subject of long debate in both human (Drevets et al., 2004) and veterinary (Low and Donachie, 1997) medicine. Experimental evidence for several independent pathways is well supported (Drevets et al., 2004). Pathways include: (i) direct hematogenous contact with endothelial cells of the

blood/brain or blood choroid layers and intracellular replication; (ii) entry of bacteria between these cells via infected phagocytes; (iii) entry into neurites (possibly delivered by macrophages) following consumption of abrasive contaminated foods and non-hematogenous transit to the brain (Low and Donachie, 1997).

CNS involvement is a particularly troublesome event. Whereas listeriae appear to replicate very well extracellularly (e.g., in cerebrospinal fluid), drugs must penetrate the blood brain barrier to have an opportunity to act. Further, some cells of the CNS that are susceptible to infection by listeriae (e.g., ependymal cells, microglial cells, and neurons (Schluter et al., 1996)) do not express MHC Class I antigens (Neumann et al., 1995) and thus the aid of T cell-mediated immunity, crucial to resolution of other forms of listeriosis, is less effective.

It is only occasionally pointed out that neurological sequelae seen in immunocompromised non-pregnant animals are rarely if ever seen in pregnant animals (Gray and Killinger, 1966). This has led some to conclude that the pregnant uterus is the only site that offers the same unbridled opportunity for replication and that the gravid host's immune system is fully competent to prevent infections elsewhere (Klink and Rudnicka, 1995; Lammerding et al., 1992; Redline and Lu, 1987).

## 5. The role of listerial cell invasion factors in pathogenesis

At the cellular level, there are a number of successive steps required for listerial infection of cultured cells (listed in the following paragraphs). Each step is defined by a listerial mutant class that fails to complete one of the steps. A more detailed description, containing extensive primary references, can be found in Dussurget et al. (2004) from which the following outline is in part based.

### 5.1. Step 1: entry

A number of cell types can be entered by listeriae and a number of listerial cell surface structures have been shown to be important in this interaction (Vazquez-Boland et al., 2001). Two of the most well-studied listerial entry factors are the products of

the *inlA* and *inlB* genes: internalin A (InlA) and InlB, respectively (Dramsı et al., 1995; Gaillard et al., 1991). Cellular receptors for each product have been identified, and the molecular signaling cascades triggered during listerial entry into host cells are being characterized in detail (Bierne and Cossart, 2002; Cossart et al., 2003). The InlA receptor is E-cadherin (Mengaud et al., 1996), a transmembrane glycoprotein normally involved in cell–cell interactions at adherens junctions of polarized cells. InlB is another member of the internalin family and its gene is located in the same operon as *inlA* (Gaillard et al., 1991). InlB is involved in entry of listeriae into a broad range of cell lines including hepatocytes but excluding epithelial cells (Dramsı et al., 1995). Mutants that have lesions in *inlA* or *inlB* show no or little attenuation (respectively) in animal models when delivered parenterally (Dramsı et al., 1995, 1997). When delivered orally, infectivity of *L. monocytogenes* strain EGD 1/2a is increased in animals (such as guinea pigs and in transgenic mice expressing human E-cadherin) whose intestinal E-cadherin has a high affinity for InlA compared to normal mouse E-cadherin (Lecuit et al., 2001). Other factors likely influence this process because mouse strains differ dramatically in systemic infectivity via the oral route (Czuprynski et al., 2003) but all have intestinal E-cadherin that matches poorly with InlA from a common laboratory strain of *L. monocytogenes* (Nelson et al., 2004). Some of this heterogeneity in susceptibility may be due to one or more alternative entry routes along the alimentary tract of mice as suggested by Lecuit (2005). However, it may be equally possible that allelic variants of *inlA*, give rise to listerial strains that are diverse in terms of InlA-binding specificity (Jeffers et al., 2001; Nightingale et al., 2005; Olier et al., 2003). Also, other factors important for cell entry and virulence via the oral inoculation route continue to be discovered (Cabanes et al., 2005; Sabet et al., 2005).

### 5.2. Step 2: escape from the uptake vacuole

Listeriolysin O (LLO), a pore forming cytolysin encoded by the *hly* gene (Mengaud et al., 1988), is the primary listerial determinant responsible for bacterial escape from the phagocytic vacuole (Gedde et al., 2000; Portnoy et al., 1988). Also, listeriae secrete two versions

of phospholipase C (PLC) that facilitate the lysis of the intracellular vacuole (Goldfine and Wadsworth, 2002). The first PLC is active for phosphatidyl inositol (PI-PLC) and is specific for phosphatidylinositol (PI) and glycosyl-PI-anchored proteins (Leimeister-Wachter et al., 1991; Mengaud et al., 1991), whereas the phosphatidylcholine (PC)-PLC has a broader substrate range (Geoffroy et al., 1991; Vazquez-Boland et al., 1992). Both enzymes act synergistically with LLO in lysing the vacuole formed when listeriae first enter a cell (the uptake [primary] vacuole) and when cytosolic replicating bacteria impinge upon adjacent cells forming a double membrane (secondary) vacuole (Camilli et al., 1993; Gedde et al., 2000). In the absence of LLO, the PC-PLC can also promote lysis of primary vacuoles in human epithelial cell lines (Grundling et al., 2003; Marquis et al., 1995). However, mutants that have lesions in *hly* are completely attenuated in vivo regardless of inoculation route (oral or parenteral (Hamrick et al., 2003)). Mutants defective for production of both phospholipases are severely attenuated in parenterally inoculated mice. However, individual elimination produces more modest attenuation (Smith et al., 1995).

### 5.3. Step 3: cytosolic replication and movement

Little is known about cytosolic bacterial growth. *L. monocytogenes* relies on the expression of a hexose phosphate transporter (Hpt) that allows bacterial intracellular replication and is required for proliferation in mouse organs (Chico-Calero et al., 2002). This permease is a structural and functional homolog of the eukaryote glucose-6-phosphate (G6P) transporter that is responsible for the uptake of G6P from the cytosol into the endoplasmic reticulum. Bacterial movement is accomplished via listerial-induced polymerization of actin filaments in the cytosol. The only bacterial factor responsible for this activity is the surface protein ActA. ActA is an envelope protein that has a transmembrane motif in its carboxyl-terminal domain that anchors the molecule to the bacterial surface (Domann et al., 1992; Kocks et al., 1992). Asymmetric distribution of ActA on the bacterial surface produces the propulsive effect of actin polymerization. Bacteria moving at the tip of actin polymerization tail can form protrusions that are engulfed by neighboring cells. Mutants with an *actA* deletion are severely attenuated

when delivered parenterally (Brundage et al., 1993). However, other *actA* mutations that more specifically impede steps in actin-based motility produce a more modest attenuation in immunologically naive mice, but in immunized mice, the defect is more pronounced (Auerbuch et al., 2001). This result may indicate that intracellular motility is of less importance to the spread of listeriae in tissues compared to the evasion of cytotoxic T cells (Portnoy et al., 2002). Additional unappreciated features of ActA may be responsible for its contribution to virulence in the naive animal.

### 5.4. Step 4: cell-to-cell spread

This stage requires the action of the two phospholipases (mentioned to in Step 2) in order for listeriae to escape the double membrane vacuole formed when the polar actin polymerization propels a bacterium from one cell into another (Mounier et al., 1990; Tilney and Portnoy, 1989). Both enzymes act synergistically with LLO in lysing this secondary vacuole (Camilli et al., 1993; Gedde et al., 2000). The lysis of the secondary vacuole brings the bacterium into the cytosol where stages 3 and 4 are repeated.

The discovery of listerial factors required for growth within cultured cells have provided microbiologists and cell biologists with many useful paradigms and tools for examining those intracellular processes (e.g., actin polymerization) and events that attend the uptake of microorganisms. However, virulence factors of *L. monocytogenes* (and in other medically important microorganisms) are defined genetically as those factors whose loss through mutation results in attenuation. This definition is very powerful provided that the mutagenesis is performed so as to produce a mutant otherwise isogenic to the parent. Whereas mistakes in this area are usually detected by genetic tests sooner or later, it is most often in the definition of attenuation that justified disagreement arises.

Attenuation depends upon a model host, a dose, and an inoculation route that have some agreed-upon relevance. Consensus with regard to these factors is seldom achieved. Indeed, some of the most well-studied listerial factors, while showing demonstrable effects in cultured cell lines, have marginal effects in vivo (Schluter et al., 1998). Further, once a mutant is shown to be attenuated, the reason for attenuation can be easily overstated. For example, work over the last 20 years has

shown the inability of *hly* mutants, deficient in listeriolysin O, to infect mice and grow intracellularly in a number of cultured cell types. In summarizing these results, it is often stated that the lack of the ability of the mutants to grow inside cells is the reason for the attenuation of *hly* mutants (Lecuit et al., 2001). However, work dating back a dozen years (Roll and Czuprynski, 1990) and more recently independently confirmed (Hamrick et al., 2003) indicates that *hly* mutants fail dramatically to colonize the intestinal tract of orally inoculated mice. Such mutants would seem to have little natural opportunity to infect host cells. Consequently, while we have learned a great deal about why *hly* and other mutants fail to multiply inside host cells (and have applied knowledge in a variety of sophisticated ways (Dietrich et al., 2001)), when speaking of the natural infection, it is unlikely that we know why *hly* mutants are attenuated. This is true in a sense for all listerial virulence factors, because the evolutionary pressures that shaped their design in this accidental pathogen are not known. For example, we recently isolated a bacteriophage resistant mutant of *L. monocytogenes* that has a cell surface alteration that prevents phage binding. The mutant is also severely attenuated both in orally inoculated mice and in its ability to grow in cultured cell lines. One would have to think that any evolutionary consequence of this lesion would be determined by the ubiquitous phage rather than the rare host. On the other hand, listeriae may naturally come into contact with predatory protozoans and may use some of the factors it has evolved in its saprophytic existence to counter a host's defenses (Ly and Muller, 1990). In this respect, it has been suggested (McLauchlin, 1997) that the listeriae may resemble some species of *Legionella* and *Burkholderia* (Greub and Raoult, 2004). These water and soil inhabiting facultative intracellular bacteria can become pathogens in the appropriately predisposed host (Inglis et al., 2004; Neumeister, 2004).

## 6. Conclusions

Listeriosis results in losses to the agricultural economy by increasing costs for production animals due to illness and increased infertility and abortion rates. Losses also accrue when consumer confidence in agricultural products is undermined: *L. monocytogenes*

regularly and decisively leads the list of bacterial food contaminants compiled by the Office of Public Health and Science (<http://www.fsis.usda.gov/OPHS/ophshome.htm>) and multistate listeriosis out breaks are relatively commonplace (MMWR 2000. 50, 1129). The ubiquity and saprophytic nature of the listeriae make it unlikely that they will be removed from the agricultural environment. However, control measures based upon early disease recognition, improvements in post-harvest monitoring, and a better understanding of host and bacterial factors that influence disease pathogenesis may combine to ameliorate the often severe and widespread effects of listeriosis. Whereas many strides have been made, the lack of certain basic information in this area (e.g., the infectious dose for humans, agreed upon in vitro tests for virulence) remain challenging. The use of *L. monocytogenes* as a tool to better understand host immunity has been very successful and many fundamental insights have accrued. Nevertheless, only recently have the roles of innate and adaptive immune responses received attention with regard to the pathogenesis of the natural disease. One practical impediment to a complete description of listeriosis is the availability of a laboratory animal that appropriately models all phases of listeriosis. Fortunately, various laboratories have turned their attention to this important problem and practical progress in this area is anticipated. Also, increased attention has been given to mimicking predisposing conditions (e.g., pregnancy) in experimental animals. This direction is important from the standpoint of human health where perinatal and neonatal listeriosis take an unnecessarily high toll. Finally, it seems ironic that after it took so long to recognize the listeriae as pathogens, their role as accidental pathogens is sometimes not emphasized. In the absence of the selective evolutionary pressure of a host, factors that make a listerial strain more (or less) pathogenic may tend to be variable. Increased emphasis on laboratory studies employing the various *L. monocytogenes* serovars is certainly warranted and an area in which listerial genomics could play an important role.

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