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Why are some *Listeria monocytogenes* genotypes more likely to cause invasive (brain, placental) infection?

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ABSTRACT

Although all isolates of the foodborne pathogen *Listeria monocytogenes* are considered to be pathogenic, epidemiological evidence indicates that certain serovar 4b lineages are more likely to cause severe invasive (neuromeningeal, maternal-fetal) listeriosis. Recently described as *L. monocytogenes* "hypervirulent" clones, no distinctive bacterial trait has been identified so far that could account for the differential pathogenicity of these strains. Here we discuss some preliminary observations in experimentally infected mice suggesting that serovar 4b hypervirulent strains may have a hitherto unrecognized capacity for prolonged *in vivo* survival. We propose the hypothesis that protracted survivability in primary infection foci in liver and spleen –first target organs after intestinal translocation– may cause *L. monocytogenes* serovar 4b hypervirulent clones to have a higher probability of secondary dissemination to brain and placenta.

KEYWORDS

Listeria monocytogenes, virulence heterogeneity, hypervirulent strains, prolonged *in vivo* survival, invasive listeriosis

Listeria monocytogenes is the causative agent of listeriosis, a foodborne infection with severe manifestations in people with weakened immunity, pregnant women and newborn infants. Clinically, listeriosis ranges from mild disease with flu-like symptoms and diarrhea to life-threatening conditions such as bacteremia and infections of the brain or placenta (1-3). The latter two are characteristic of the invasive form of the disease and are respectively known as central nervous system (CNS) or neuromeningeal listeriosis, typically in the form of meningoencephalitis, and maternofetal/neonatal (MFN) listeriosis, presenting as miscarriage, stillbirth or neonatal sepsis (4). Listeriosis is of great concern to the food industry due to the frequent occurrence of outbreaks and the cost of product recalls and food-safety measures (5). An important issue is that regulatory authorities consider all *L. monocytogenes* strains as pathogenic, whereas only a few genotypes cause most listeriosis cases (6-8). There is therefore a pressing need to better understand *L. monocytogenes* diversity and its relationship with pathogenicity in order to target food safety interventions only to products contaminated by hazardous strains. Recent findings from integrated analysis of *L. monocytogenes* population genetics and epidemiological/clinical data (9) (see below) make the time ripe to discuss some unpublished observations from our laboratory that may help guiding further research into this topic.

***L. monocytogenes* diversity and virulence heterogeneity.** *L. monocytogenes* is a slow evolving yet diverse species that can be grouped into four major evolutionary lineages (I to IV), 13 lineage-related serovars (sv), and >100 clonal complexes (CC) defined by multilocus sequence typing (MLST) and whole-genome phylogenetic analysis (6, 10-14). While all strains of the species are potentially pathogenic, a wealth of epidemiological evidence indicates that it is pathogenically heterogeneous. Thus, only three of the 13 *L. monocytogenes* serovars, i.e. 4b and 1/2b within lineage I, and 1/2a within lineage II, are implicated in over 95% of human listeriosis cases (1, 2, 15). Comparative analyses of isolates from food surveys and clinical specimens (human or animal) also demonstrate an uneven distribution, with lineage II strains predominating

in the former (chiefly sv 1/2a and sv 1/2c) and lineage I sv 4b strains in the latter (8, 16). Moreover, specific sv 4b clones, namely CC1, CC2, CC4 and CC6, are overrepresented among clinical isolates and epidemic strains (9, 16), and tend to be isolated from patients with fewer or no immuno-compromising co-morbidities (9). At the other side of the spectrum, certain lineage II clones, such as CC9 and CC121, are strongly associated with a non-clinical (food) origin or, if causing infection, with highly immunocompromised patients (9). Consequently, the sv 4b CC1, CC2, CC4 and CC6 clones have been considered as "hypervirulent", the "food-associated" CC9 and CC121 as "hypovirulent", and the rest of prevalent *L. monocytogenes* CCs as "intermediate" (9). Interestingly, both CNS and MFN listeriosis are statistically associated with the hypervirulent *L. monocytogenes* clones, particularly CC1 and CC4, in contrast to the hypovirulent clones CC9 or CC121, which are associated with bacteremia with no CNS or MFN involvement (9). Collectively, these observations support the notion that the *L. monocytogenes* hypervirulent clones may possess specific attribute(s) that facilitate brain or placental infection.

Basis of *L. monocytogenes* "hypervirulence": an elusive question. *L. monocytogenes* hypovirulence has been linked to virulence gene polymorphisms leading to attenuation (17, 18), notably mutations in the *inlA* gene which result in a truncated form of the invasion-associated protein InlA (9, 19). These *inlA* mutations are observed in 25-50% of lineage II food isolates and correlate experimentally with impaired entry into non-phagocytic cells (e.g. epithelial cells), offering a plausible explanation to the hypovirulent phenotype. On the other hand, pangenome studies have identified a number of accessory virulence-associated genes as specific to the hypervirulent (CC1, CC2, CC4 and CC6) clones (7, 9). Examples include the listeriolysin S gene cluster (LIPI-3) (20), sv 4b-specific teichoic acid biosynthetic genes (21), or a cellobiose family phosphotransferase system (PTS). Deletion of the latter has been reported to result in decreased CNS and fetal infection in mice (9), but it is only present in CC4 isolates, not in the other hypervirulent CCs. Other studies found two members of the internalin multigene family, InlF and Lmo2470 (InlP), to be involved in brain invasion (22) and placental tropism (23),

53 respectively. However, both InlF and InlP are conserved across different *L. monocytogenes*
54 lineages and therefore are unlikely to play a significant role in the differential pathogenicity
55 exhibited by some sv 4b CCs. Whether any of the above genetic determinants are actually
56 mechanistically involved in *L. monocytogenes* tropism for brain and/or placenta requires
57 additional investigation. To date, a clear differential functional marker that could be linked to *L.*
58 *monocytogenes* "hypervirulence" (understood as an increased ability to cause invasive infection)
59 has not been identified.

60 **Prolonged *in vivo* survival of hypervirulent serovar 4b strains**. Preliminary data from
61 mouse experiments in which we monitored listerial survival in organs beyond the typical
62 standard 5 to 7 day time-course, i.e. up to 20/21 days post infection, may offer some clues (Fig.
63 1). In these experiments, BALB/c mice were infected intravenously (i.v.) with four different *L.*
64 *monocytogenes* isolates (Table 1). (i) PF49, the epidemic strain of a cheese-associated outbreak
65 in Switzerland where 79% of cases were CNS infections (24). (ii) P14 isolated from an adult
66 patient with CNS manifestations during a listeriosis outbreak in Spain (25). Both P14 and PF49
67 belong to the sv 4b hypervirulent clonal complex CC1. (iii) G6006 of sv 1/2b, responsible for an
68 outbreak of febrile gastroenteritis due to chocolate milk in USA where none of the 45 affected
69 people developed invasive listeriosis (26). This same strain was recovered from additional cases
70 in the community most of which were also non-invasive infections (febrile gastroenteritis $n = 5$,
71 bacteremia $n = 2$; only one CNS infection in a 72-year-old with several co-morbidities) (26).
72 G6006 belongs to clonal complex CC3, which comparatively is much less frequently found
73 among clinical isolates, is not statistically associated with invasive listeriosis, and is classified in
74 the "intermediate virulence" category (9). And (iv) the reference genome strain EGDe (27), of sv
75 1/2a, widely used as experimental model in *L. monocytogenes* pathogenicity studies (28). EGDe
76 was supposedly a derivative of the sv 1/2a EGD strain used by Mackaness in his pioneering
77 studies on cell-mediated immunity (29), in turn assumed to be one of the original isolates of
78 E.G.D. Murray et al. who first identified *L. monocytogenes* in 1924 (30); however, EGDe has

been later shown to be genomically unrelated to EGD (28) and its origin is uncertain. EGDe belongs to the food-associated hypovirulent clone CC9, very rarely associated with clinical listeriosis (9). While EGDe exhibits the normal virulence features of *L. monocytogenes* in standard *in vitro* and *in vivo* experiments, it has been found to be poorly neuroinvasive in a mouse infection model (9). All four strains were confirmed to be wild type, including a wild-type *prfA* genotype with the usual virulence-related functional characteristics (31).

EGDe and G6006 displayed the expected behavior of *L. monocytogenes* in the organs of i.v. infected naïve wild-type mice (Fig. 1). After a systemic infection, a progressive decrease in bacterial numbers is typically observed between days 3 to 7 until complete clearance by day 10 p.i. (32-35) as a consequence of effective macrophage activation and protective Th1 and CD8+ T-cell responses (36, 37). A similar pattern was exhibited by the sv 4b strains up to day 10 p.i., albeit with generally higher bacterial numbers, particularly in the liver. Strikingly, however, after virtual disappearance by day 14/17 p.i., the sv 4b bacteria were again recovered in significant numbers at day 20 or 21 for both PF49 and P14 in the liver, and P14 in the spleen (Fig. 1).

The fact that both neurolisteriosis-associated isolates, PF49 and P14, exhibited the same behavior suggests that a capacity for prolonged *in vivo* survival might be a distinctive feature of the hypervirulent sv 4b strains compared to other *L. monocytogenes* genotypes. This ability has so far remained unnoticed because *L. monocytogenes* virulence studies have been historically (and currently still are) based on model strains of sv 1/2a like EGDe or 10403S (28). Based on the abundant historical data with sv 1/2a model strains, listerial full clearance from liver and spleen 7-10 days p.i. is the accepted dogma in systemically (i.v.) infected mice. Accordingly, most *in vivo* mouse studies with *L. monocytogenes* are generally limited to short infection time-courses below five to seven days (see e.g. for recent examples [9, 38]).

Implications for pathogenesis. In the framework of our understanding of listeriosis pathophysiology (1) (Fig. 2), a prolonged *in vivo* survivability affords a reasonable explanation of why certain *L. monocytogenes* strains are more often associated with invasive infection.

Listeria infection begins with bacterial crossing of the intestinal barrier and translocation to the primary target organs, i.e. the liver and spleen (1). In immunocompetent individuals, these initial stages are generally subclinical and self-limiting (unless a high *L. monocytogenes* dose is ingested, in which case febrile gastroenteritis may develop a few hours after ingestion of the contaminated food [39]). However, inadequate containment of the primary infection foci results in bacterial release into the bloodstream (bacteremia is indeed often observed in the course of listeriosis [4]) and dissemination of *L. monocytogenes* to the secondary target organs, i.e. the brain in immunocompromised adults or elderly people and the placenta in pregnant women (1, 40) (Fig. 2). Except for the ascending intra-axonal invasion of the rhombencephalon from oropharyngeal cranial nerve terminals, evoked in ruminants and occasionally in people (1, 41), neurolisteriosis generally results from hematogenous invasion of the brain (42, 43). In systemically infected mice, listerial brain invasion has been shown to critically depend on the level and duration of bacteremia (35). Studies in systemically infected pregnant guinea pigs also concluded that MFN listeriosis results from small numbers of *L. monocytogenes* bacteria trafficking from the maternal organs to the placenta (44). It can therefore be safely assumed that an ability for sustained survival at the primary infection sites in liver and spleen can directly translate into an increased likelihood of successful secondary dissemination of *L. monocytogenes* to the CNS or placenta (Fig. 2). This notion is consistent with the relatively long incubation period of CNS and MFN listeriosis, of up to 14 to 67 days (45), showing that the development of invasive listeriosis clearly depends on a previous protracted host-pathogen interaction process which implies prolonged bacterial survival.

Concluding remarks. We provide here an initial insight into a previously unrecognized virulence phenotype that offers a working hypothesis about why *L. monocytogenes* hypervirulent CCs may be more commonly associated with invasive listeriosis (Fig. 2). Further investigations should aim at systematically comparing the *in vivo* behavior of hypervirulent, hypovirulent and intermediate CC strains (9), and to ascertain whether prolonged survival in

primary infection foci in the liver and spleen results in increased hematogenous spread to brain and placenta. Our experiments were limited to a time-course of 20/21 days and it would be important to determine the duration of the *in vivo* survivability of *L. monocytogenes* and its relationship with bacteremia. During listeriosis, bacteremia occurs with or without invasive infection; indeed it is the clinical manifestation most commonly seen with hypovirulent CCs (9). Since hypovirulent CCs are typically found in highly immunocompromised patients or with significant co-morbidities (9), the association of these CCs with bacteremia may simply be a reflection of the early application of diagnostic blood cultures (systematically performed whenever a febrile process is detected in this vulnerable patient cohort) before invasive (brain) infection can develop. Alternatively, hypervirulent strains could possess specific attributes, in addition to a prolonged *in vivo* survivability, that would promote brain and/or placental invasion. Further research should determine whether the hypervirulence of sv 4b CCs involves the presence/absence (or differential expression) of specific bacterial genetic determinants, as well as potential mechanism of immune evasion or manipulation of host responses.

ACKNOWLEDGMENTS

This work received support from the Spanish Ministry for Science and Technology (grant BMC2000-0553) and the Welcome Trust (WT074020MA). M.W. was supported by a Schrödinger visiting fellowship from the Austrian Research Fund (J-1694).

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TABLE 1. *L. monocytogenes* strains.

Strain [a]	Serovar	CC [b]	Source / description	Clinical manifestation	Reference
PF49	4b	CC1	Epidemic strain of cheese-associated outbreak, Vaud (Switzerland) 1983-1987	Neuromeningeal	(24, 46)
P14 (PAM 14)	4b	CC1	Listeriosis outbreak, Valencia (Spain) 1989	Neuromeningeal	(25, 31, 47)
G6006 (FSL-R2-0597)	1/2b	CC3	Epidemic strain of chocolate milk-associated outbreak, Illinois (USA) 1994	Non-invasive (febrile gastroenteritis)	(26, 46)
EGDe	1/2a	CC9	*L. monocytogenes* reference genome (T. Chakraborty)	Unknown	(9, 27, 28)

[a] Other designations in brackets.
[b] Clonal complex.

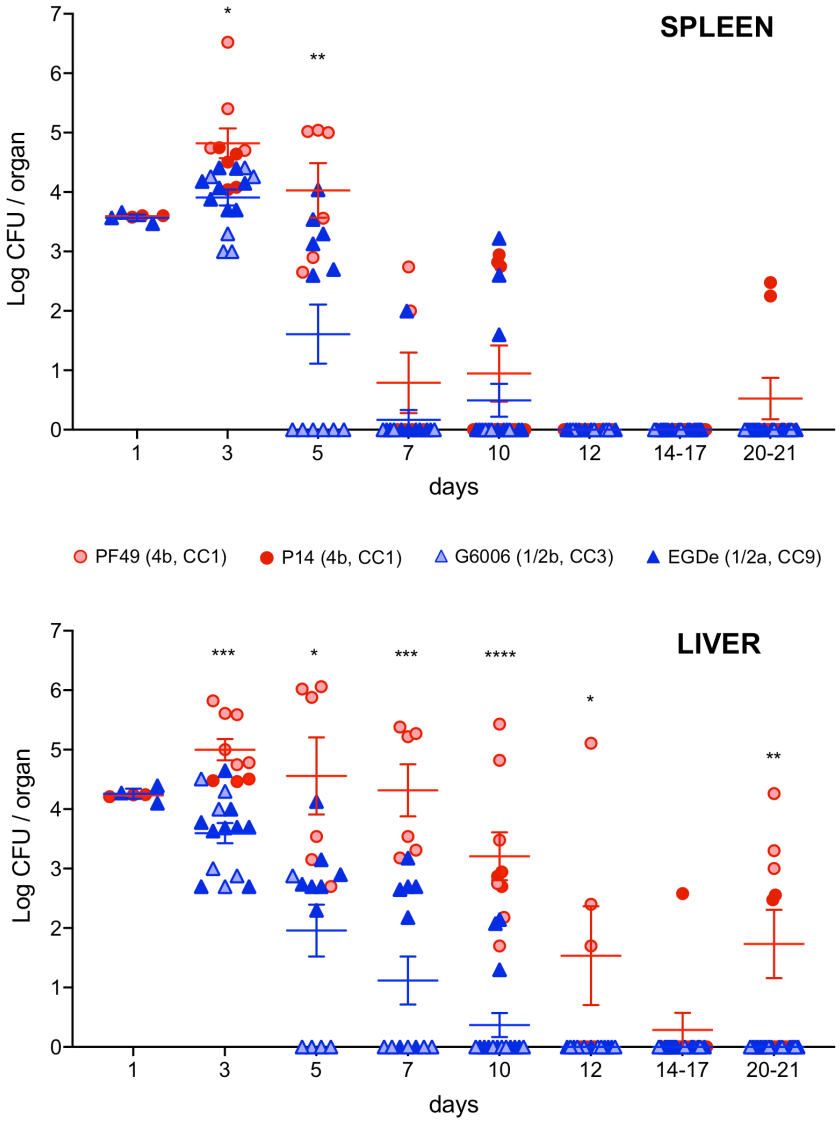

FIG 1. Prolonged *in vivo* survival of *L. monocytogenes* sv 4b (CC1) strains. Groups of 6- to 8-week-old BALB/c female mice (three per group) were infected via the tail vein with 3 to 5 × 10^3 CFU per animal. At the indicated time points, mice were euthanized, spleens and livers recovered and homogenized, and bacterial numbers determined by serial dilution and plate counting in BHI agar. Experiments were performed at Universidad Complutense de Madrid (two series with strains PF49, G6006 and EGDe) and University of Edinburgh (additional series with strains P14 and EGDe). Each symbol represents a mouse. Data for mice infected with the sv 4b/CC1 (neuromeningeal infection) strains PF49 and P14 are shown in red symbols, in blue symbols those for strains G6006 and EGDe. Line diagrams in corresponding color represent the combined mean ± SEM for each of these two categories, with statistically significant differences indicated on top (two-way ANOVA and Fisher´s Least Significant Difference test; *P* values: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001). Experiments were conduced according to applicable regulations and guidelines in animal experimentation (Complutense University: animal facility registration no. 28079-I5ABC-M, Real Decreto 223/1988, Orden 13/10/1989, EU Directive 86/609/CEE; Edinburgh University: UK Home Office project licence under the 1986 Animals [Scientific Procedures] and approval by local Ethical Review Committee).

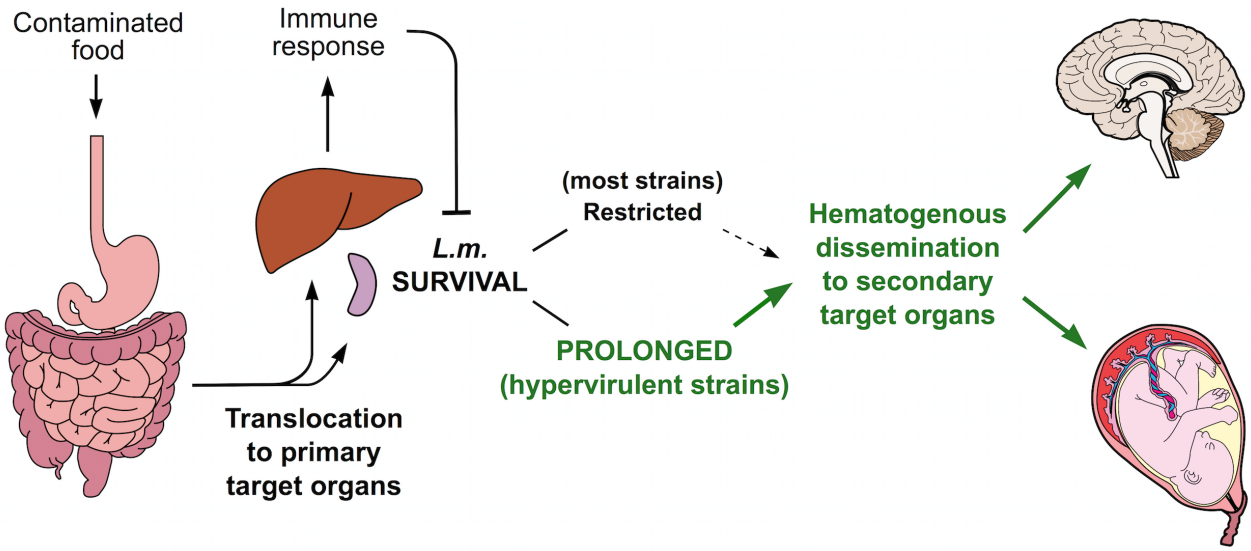

FIG 2. Model illustrating the hypothesis that prolonged survivability in primary infection foci in liver and spleen may explain the increased likelihood *L. monocytogenes* serovar 4b hypervirulent strains to cause brain and placental infection. Schematic of the pathophysiology of invasive listeriosis modified from original diagram in ref. (40); see explanations therein and in ref. (1) for details.