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Foci of *Listeria monocytogenes* persist in the bone marrow

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SUMMARY

Murine listeriosis is one of the most comprehensive and well-studied models of infection, and *Listeria monocytogenes* has provided seminal information regarding bacterial pathogenesis. However, many aspects of the mouse model remain poorly understood, including carrier states and chronic colonization which represent important features of the spectrum of host-pathogen interaction. Bone marrow has recently been shown to harbor *L. monocytogenes*, which spreads from this location to the central nervous system. Bone could, therefore, be an important chronic reservoir, but this infection is difficult to study because it involves only a few bacteria and the extent of infection cannot be assessed until after the animal is sacrificed. We employed in vivo bioluminescence imaging to localize *L. monocytogenes* bone infections over time in live mice, revealing that the bacteria grow in discrete foci. These lesions can persist in many locations in the legs of mice and are not accompanied by a histological indication such as granuloma or a neutrophil infiltratate. We demonstrate that highly attenuated *hly* mutants, which have defective intracellular replication, are capable of prolonged focal infection of the bone marrow for periods of up to several weeks. These results support the recently proposed hypothesis that the bone marrow is a unique niche for *L. monocytogenes*.

INTRODUCTION

Listeria monocytogenes, the causative agent of listeriosis, is a multifaceted pathogen that infects mainly immunocompromised people involving a complex interaction between bacterium and host. In humans, this bacterium spreads to many organs and can cause a lethal septicemia, particularly in newborns. *L. monocytogenes* elicits strong cellular immune responses (Yoshimura et al., 2006) and is currently in clinical trials as an anti-cancer vaccine. Human organs that can be infected by *L. monocytogenes* include the brain and spinal cord (Frayne and Gates, 1987; Leiti et al., 2005; Mylonakis et al., 1998), the liver (Marino et al., 1996; Yu et al., 1982) and the placenta (Parkash et al., 1998). There are currently fewer than 20 instances of human osteomyelitis caused by *L. monocytogenes* that have been reported in the searchable literature (Khan et al., 2001). Although very rare, these cases demonstrate that *L. monocytogenes* can infect the bone in humans and that this process also occurs in many of the animal models of listeriosis. For example, *L. monocytogenes* reproducibly infects the knee joints of experimentally infected birds (Huff et al., 2005). Mice serve as an excellent model for resolving the molecular and cellular features of *L. monocytogenes* infection because of the well-studied biology of the murine system. Murine listeriosis has generated a wealth of information regarding pathogenesis and the mouse model continues to be a stalwart of immunology.

L. monocytogenes can be cultured from the bone marrow of infected mice (de Bruijn et al., 1998) and has been shown to infect myeloid cells of the surface phenotype: $CD31^+$, $Ly\text{-}6C^+$, $CD11b^+$ (Join-Lambert et al., 2005). These cells are found in the bone marrow and the blood, and represent an important reservoir of the pathogen with regard to the infection of the central nervous system. The bacteria can be cultured from the bone marrow even after modest inocula of 3×10^3 CFU (colony forming units) (de Bruijn et al., 1998). In contrast, large intravenous (i.v.) inocula of 10^7 CFU were required to observe the bacteria in the bone marrow under the microscope, and less than 5% of bone marrow cells were infected at 24 hours after i.v. injection of 2×10^7 CFU (Join-Lambert et al., 2005), which is roughly 1000 times the dose required for 50% lethality (LD_{50}). Thus, this infection can be detected by culturing the bacteria but is not readily observed when using doses similar to the LD_{50}, or in sublethal infections. The infection of bone marrow was not reported to exhibit any outward symptoms in the animal. Exactly which bones are infected, and the location and extent of the infection in a given bone, remains impossible to determine in any animal by gross inspection. Studies have been limited to selected bone marrow samples excised from animals that were sacrificed at predetermined time points; therefore, they cannot address the course of infection in any one animal or assess the bacterial load in bones that are not selected for dissection. Thus, we have only a limited view of this infection process. Whereas infected cells in the bone marrow have been well characterized with regard to lineage, and the subsequent infection of the central nervous system by this subset of cells has been established, the distribution of bacteria in the bone marrow of the whole animal has not been determined. Because in vivo bioluminescence imaging (BLI) can detect a few thousand bacteria, non-invasively, in the most internal tissues of live mice (Contag et al., 1995; Hardy et al., 2004), we employed this technique to analyze the colonization of bone by *L. monocytogenes* in order to reveal the spatial growth patterns and kinetics of this interesting infection over time.

BLI is a method in which cells, genes or organisms are genetically tagged with light-producing luciferase enzymes which generate signals that can be detected through the tissues of live anesthetized animals using an ultra-sensitive charge-coupled device camera (Contag et al., 1995). When employed with *L. monocytogenes* that

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has been genetically labeled with an integrated bacterial *lux* operon, this technique revealed signals emanating from the legs of many infected animals. Here, we describe experiments demonstrating that the leg infection is focal and that foci occur in the bone after oral or i.v. inoculation. The focal infection of hind limbs, including phalanges, occurred in both susceptible BALB/c and resistant outbred CD1 animals with the virulent strain of *L. monocytogenes*. The mice exhibiting such signals showed no outward sign of distress, and no inflammation of infected areas could be discerned. When infected leg bones were excised and imaged, the bones exhibited focal signals indicating that the infection was not present in soft tissues. The bacteria infecting the legs were located in the bone marrow, as determined by the recovery of CFU from that compartment, which is consistent with previous studies (de Bruijn et al., 1998; Join-Lambert et al., 2005). However, no granulomatous lesions or other indications of inflammation were discernable in the infected bones by histology. Surprisingly, foci of bone infection by highly attenuated *L. monocytogenes* mutants, with compromised intracellular replication and cell entry, sometimes persisted for several weeks, which was not observed in other locations within the body. Thus, the host-pathogen relationship in the bone marrow is apparently distinct from other tissues such as the liver, the intestine and the spleen, which did not show persistence of attenuated strains and did not display persistent isolated focal signals.

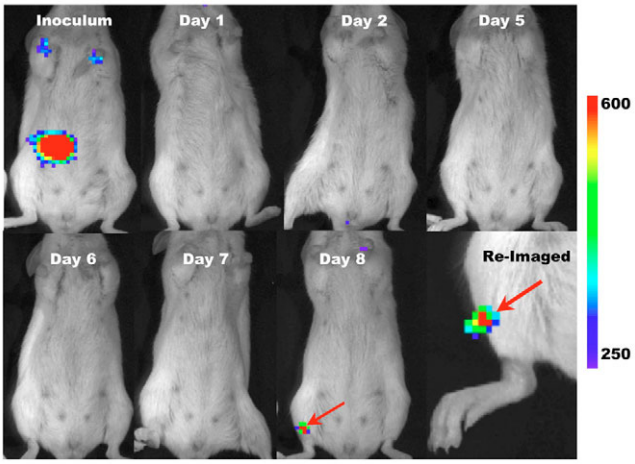

Fig. 1. Focal infection of the leg after oral inoculation of *L. monocytogenes*. BALB/c mice were infected orally with 2×10^9 CFU of the virulent bioluminescent 2C strain of *L. monocytogenes* and imaged each day (the day of inoculation is designated day 0). One of five animals is shown, on selected days. The bacteria were visible in the abdomen from 30 minutes post-inoculation (A). The mouse shown developed a signal from the leg on day 8 (arrow), so it was re-imaged at a closer setting. The color bar indicates photon counts of BLI signal per pixel over the 5-minute integration time for all images. Diffusion of the light is apparent, as some of the signal appears to emanate from outside the limb. The signal was gone the next day.

RESULTS

Signals from the tibia and other bones

The infection of mice by virulent *L. monocytogenes* can result in a wide range of severity and tissue tropism, from rapid and fatal disease to asymptomatic clearance. We, and other groups, have previously characterized the BLI patterns associated with lethal and sublethal infections by this bacterium (Hardy et al., 2004; Hardy et al., 2006; Riedel et al., 2007). Dynamic signals from the liver, spleen, lymph nodes, brain, kidney and other organs have revealed the complex interaction between *L. monocytogenes* and the host. Upon oral infection using 2×10^9 CFU of the virulent 2C strain, BALB/c mice usually clear the bacteria in the first 12 hours. Signals can reappear in the gallbladder and other organs after a few days (Hardy et al., 2004), and usually occur in the absence of symptoms. At higher oral doses, lethal illness occurs in some animals. We observed focal BLI signals first appearing from the leg as many as 8 days after initial clearance of oral infection (Fig. 1). In this case, the mouse shown was completely asymptomatic throughout the experiment and did not generate signals from any other locations after clearing the inoculum.

Intravenous infection also resulted in persistent focal BLI signals from the legs. Images revealing multiple lesions in animals displaying no apparent symptoms (Fig. 2A) prompted us to investigate further the location and genetic requirements of this growth pattern. We used BLI to ascertain the location of the infection in individual animals over time and to direct ex vivo assays to investigate the foci. In addition to hind legs, focal signals are also observed in other bones such as the phalanges (Fig. 2B). Interestingly, the front limbs rarely showed signal. All of these infections occurred without any outward indication, such as redness or swelling, and without any apparent effect on the mobility of the animal. In the animal shown in Fig. 2B, the signal persisted for 5 days.

Attenuated mutants

To investigate the genetic basis of this infection, we sought first to determine the role of the *L. monocytogenes* gene *hly*, encoding the pore-forming listeriolysin O (LLO) protein that lyses the phagocytic vacuole and permits intracytoplasmic growth in macrophages and other cells (Portnoy et al., 1988). This gene is crucial for virulence. The *hly* deletion mutant of the *L. monocytogenes* 2C strain that we use is five orders of magnitude less virulent than the parent strain. Additionally, we examined the role of *prfA*, the master positive regulator of *hly* and other virulence factors (Chakraborty et al., 1992), and examined mutations in the genes encoding the A and B internalins, *inlA* and *inlB*, which encode cellular uptake proteins (Gaillard et al., 1991). Mice infected with all of these mutants display signals from the leg after i.v. administration of lethal and sublethal doses (Fig. 2C,D; Fig. 3). The minimum doses of these attenuated strains that are necessary for observing leg signals are those that cause animals to begin to exhibit symptoms such as matted fur and conjunctivitis on day 1 and/or day 2 before recovering (5×10^8-1×10^9 CFU). In BALB/c mice, using 1 LD_{50} doses of the attenuated strains (2×10^9 CFU), two or three animals in each group of five usually displayed leg signals. Further, when attenuated mutants are administered intravenously at sublethal doses, leg signals are evident in many animals. As an example, a single experiment is shown in Table 1. The wild type 2C mice also exhibit leg signals at many doses. In the experiment that led to the data in Table 1, a dose of 1 LD_{50} was administered to the wild-type 2C mice.

Persistence of attenuated mutants

Attenuated mutants that do not express LLO do not induce protective immunity and are readily cleared from the liver and spleen (Muraille

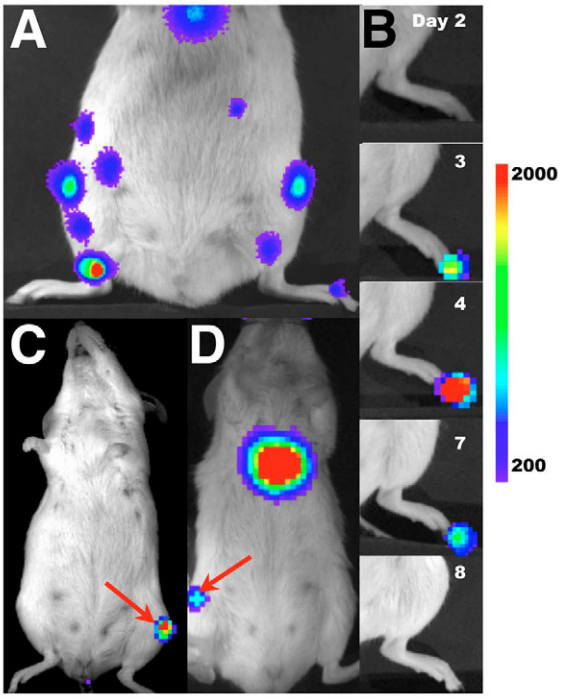

Fig. 2. Leg signals from virulent *L. monocytogenes* and attenuated mutants after i.v. infection. (A) A CD1 mouse infected intravenously with the virulent 2C strain of *L. monocytogenes* shows multiple signals from the lower limbs on day 4. (B) A CD1 mouse infected with the virulent strain showing that signals were detected from the phalanges on the indicated days. An *hly* mutant [a BALB/c female shown on day 3 (C)] and a *prfA* mutant [a BALB/c female is shown on day 2 (D)] can also colonize the leg. In the experiments shown, the numbers of mice exhibiting leg signals in each group were: (A), *n*=5/5; (B), *n*=1/5; (C), *n*=3/5; and (D), *n*=1/3. The large circular signals over the lower thorax in (A) and (D) are from the gallbladder.

et al., 2005; Portnoy et al., 1988). However, these mutants are capable of extracellular replication in the lumen of the gallbladder for weeks (Hardy et al., 2004; Hardy et al., 2006). Therefore, we sought to determine how long the leg signals produced by these mutants persisted. Surprisingly, we found that administration of an *hly*, *inlA*, *inlB* triple mutant, at lethal and sublethal i.v. doses above 10^9 CFU, resulted in prolonged colonization of the tibia (Fig. 3) in approximately one or two mice in each group of five animals. The location of this signal, which was frequently observed with both virulent and attenuated strains, was apparently between the joints, consistent with infection of the bone rather than the joint. In the experiment shown, the signal was still present in two animals on day 24 (Fig. 3B, the surviving cohort after 1 LD_{50} doses is shown), and on day 27 the animal with the strongest signal was sacrificed and the tibias removed for ex vivo imaging (Fig. 3D). The location of the signal in the bone corresponds to the junction between the tibia and fibula, which are fused distally in mice. Cultures from the marrow of the bone on the left in Fig. 3D yielded 7000 CFU, which is a remarkably small number given the signal detected. The signal intensity from the bacteria in this location in the live animal was 3.1 photons per CFU per second, which is 30 times that observed in the gallbladder (Hardy et al., 2004). A greatly increased signal was obtained by removing the small amount of overlaying tissue and excising the bone. Upon sacrifice of the mouse and excision of the bone, the number of photons per second (calculated to compensate for differing exposures) increased from 1.6×10^4 photons per second in the live animal to 1.0×10^5 photons per second for the mouse's right leg and from 2.3×10^4 photons per second to 1.8×10^5 photons per second upon excision of the bone for the mouse's left leg. The readings were obtained from a 1 cm^2 circle around the region of interest, which, for the excised bones, was determined based on the signals in the live animal. In addition, the increased photon scattering resulting from the overlying tissue is evident in the broader signal area observed in the live animal relative to that of the excised bone.

Histology

Because BLI permits the localization of signals to a given part of the bone, image-guided histology might reveal the associated lesion. Lesions owing to *L. monocytogenes* very often involve granulomas and/or neutrophil infiltrates that are readily observed in the spleen and liver using standard staining techniques (Marino et al., 1996). *Staphylococcus aureus* causes granulomatous bone marrow lesions and accompanying pathology that are very apparent in histological

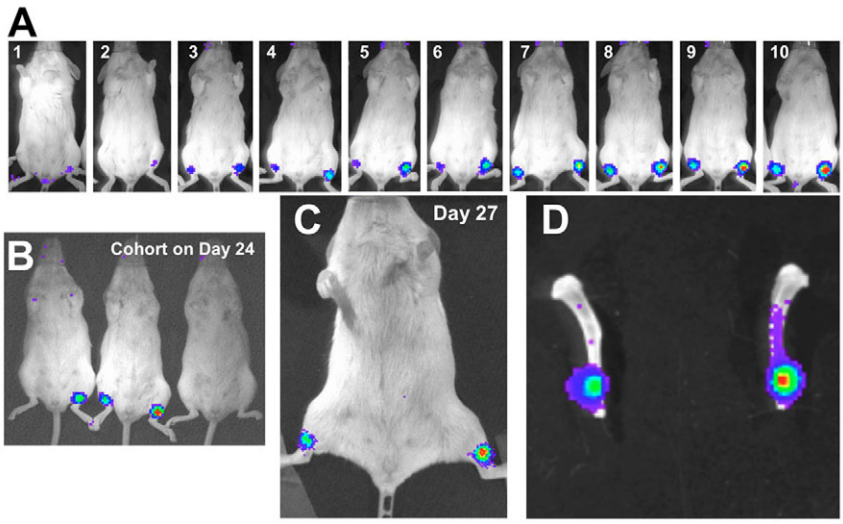

Fig. 3. Persistence of attenuated mutants in the bone marrow. BALB/c mice were inoculated intravenously with 2×10^9 CFU (1 LD_{50}) of the attenuated *L. monocytogenes* strain 2C *hly*, *inlA*, *inlB* and imaged each day. (A) Two out of five mice exhibited signals from the area of the leg corresponding to the tibia, beginning on day 1. One of these two mice is shown for the first 10 days. (B) The signal was still present at day 24 in two of the three surviving animals. (C) On day 27, the mouse showing two leg foci was imaged at higher resolution with its legs extended. The mouse was sacrificed and the tibias removed and imaged ex vivo (D). The bone on the left yielded 7000 CFU of bioluminescent *L. monocytogenes* from the marrow. A 1 cm circular region over the corresponding signal from the animal before it was sacrificed (not shown) recorded 2.2×10^4 photons/second, representing a value of 3.1 photons detected/second for each CFU recovered.

Table 1. Duration of signals from three strains of listeria at different organ sites in individual animals

Strain:	2C*Δhly*					2C					2CΔp60				
Dose:	1e+08 CFU (0.1 LD_{50})					4e+04 CFU (1 LD_{50})					1e+06 CFU (0.5 LD_{50})				
	Animal number					Animal number					Animal number				
Day	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	G,N,**L**	0	N,**L**	**L**	I	0	N	G,N	G,S	I	G	S	G,I	G	G,**L**
3	**L**	0	N,**L**	G,I,**L**	0	G,I	0	G	G	G,N,**L**	G,N	G	G,**L**	G	G,**L**
4	G,**L**	0	N,**L**	0	0	G,I	G,I	G,I,N	G,I	G,N,**L**	G,N	GI	G,**L**	G	G,**L**
5	G	0	N,**L**	G	0	G,I	G,I	G,I,S,H	I	G,N,**L**	G,N	G	G,**L**	G	G,N
6	G	0	**L**	G	**L**	G,I,S	G,I	X	I,H	G,**L**	G	G	G,**L**	G	G
7	G	0	**L**	G	**L**	G,I,S	G		X	G,**L**	G	G	G,**L**	0	G
8	G	0	**L**	0	I,**L**	G,I,S	N			G,**L**,S	G	G	G,**L**	0	I
9	G	0	0	0	0	G,I,S	G,I,N			G,**L**,S	N	0	G,**L**	0	G
10	G	0	0	0	0	X	G,I,N			G,S	0	X*	G	0	G
11	G	0	0	0	0		G,I,N			G,S	0		G	0	G
12	G	0	0	0	0		G,I,N			N,S	0		0	0	0

0=no detectable signal; G=gallbladder; N=lymph node; **L**=leg; I=intestine; S=spleen; H=liver; X=moribound and euthanized; X*=dead (this animal exhibited no signal on the day before death; whereas the other animals exhibited high signals before euthanasia). Days 1-12 after infection are indicated in the 'day' column on the far left.

sections (Matsushita et al., 1997). To determine if *L. monocytogenes* caused similar lesions, we excised leg bones from mice displaying BLI signals (not shown) for image-guided histology (Fig. 4A,B). These sections were compared with uninfected bone. No difference was discernable between infected and uninfected samples, and no granulomatous lesions or neutrophil infiltrates were observed, even with signals indicating the presence of many tens of thousands of bacteria. This result is in apparent contrast to organs such as the liver and spleen, which exhibit pronounced histological indications of *L. monocytogenes* infection when signals are present. Upon tissue Gram stain, no intracellular or extracellular bacteria were evident (Fig. 4B). This result is not surprising, as *L. monocytogenes* is difficult to observe by microscopy in dense tissues with high cellularity, such as the spleen and liver, unless very large numbers of bacteria are present (Join-Lambert et al., 2005; Muraille et al., 2005). Although *L. monocytogenes* cells can be observed in bone marrow tissue sections, this requires the use of large inocula (Join-Lambert et al., 2005).

L. monocytogenes exhibits strain differences with regard to infection. To determine whether the focal bone infection and lack of obvious histological signature were specific to the *L. monocytogenes* strain that we generally employ (10403S), a *lux*-labeled *L. monocytogenes* strain EGDe (Corr et al., 2007) was injected intravenously into five female CD1 mice for BLI-guided histology. In this experiment, on day 4 post-infection, one of the mice showed a strong signal from the leg, apparently from the joint (Fig. 4C). This mouse was sacrificed and the intact joint was excised and imaged (Fig. 4D). The signal appeared to be associated with the proximal terminus of the tibia rather than inside the synovial fluid of the joint. The location of the signal was revealed best by using the fade function of the imaging software (as shown), which creates a translucent pseudo-color overlay so that the tissue can be visualized more clearly. The joint was sectioned and stained with tissue Gram stain, showing that the two bones, uninfected (Fig. 5E) and infected (Fig. 5F), were indistinguishable with regard to lack of pathology, and that no granuloma or neutrophil infiltrate was apparent. Bone destruction was not observed with trichrome staining (Fig. 4G, infected bone shown), which is used to assess bone morphology and integrity.

DISCUSSION

The use of BLI for the analysis of *L. monocytogenes* in the body provides a rapid and accurate means of determining sites of bacterial replication because light is only emitted at detectable levels by growing cells in early log phase (Hardy et al., 2004). Dying or dormant bacteria are not detectable so the signals are derived from locations in which the bacteria are growing, indicating sites of active infection. The technique also permitted us to differentiate between bacteria that were present in the bone marrow as a result of circulating infected $CD31^+$, $Ly\text{-}6C^+$, $CD11b^+$ myeloid cells from the blood and bacteria that were replicating and remaining in situ. This distinction was possible because the latter were growing in discrete foci rather than in a diffuse manner. In some cases, the foci lasted many days indicating that the particular focus was stable with regard to position and implying that the bacteria remained largely within a given focus. These results are consistent with models of listerial bone marrow infection that suggest that this compartment serves as a distinct replication site (de Bruijn et al., 1998; Join-Lambert et al., 2005). This reservoir may ultimately seed the central nervous system with bacteria.

Signals from the leg are common in our imaging experiments with *L. monocytogenes* and demonstrate that this organism readily infects this tissue type in highly resistant mice (CD1 outbred mice), as well as more susceptible mouse strains (BALB/c). The bone marrow contains many circulating cells including neutrophils that should be capable of eliminating *L. monocytogenes* and yet foci appear even 8 days after oral clearance. Upon oral infection, *L. monocytogenes* classically enters a latent period followed by re-emergence some days later, which has been confirmed by BLI (Hardy et al., 2004). Thus, the apparent disappearance and re-emergence of this pathogen after oral inoculation is not unprecedented. However, the location of the bacteria during this latent period is unknown and the re-emergence of the signal in

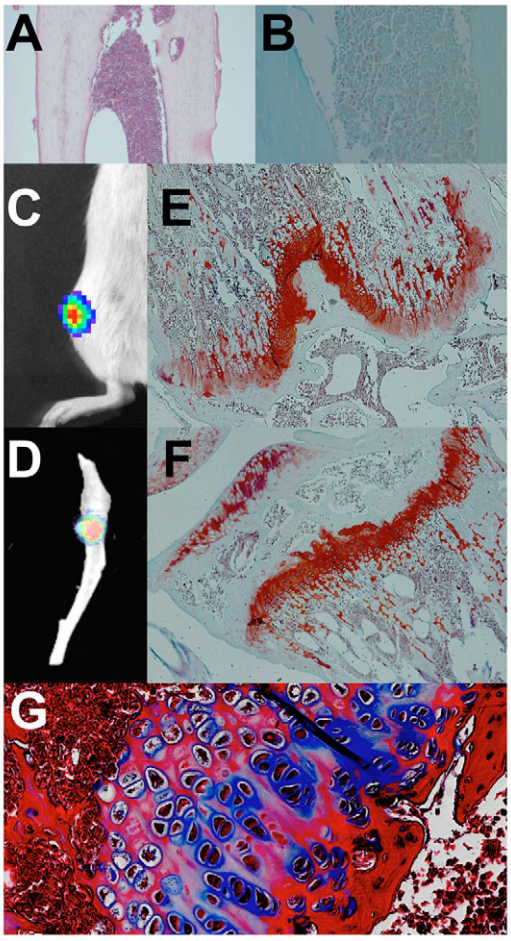

Fig. 4. Histology of infected bone. BALB/c mice were inoculated intravenously with 8×10^4 CFU (2 LD_{50}) of the *L. monocytogenes* strain 2C and imaged each day. When focal bone signals appeared in their legs (not shown) the animals were sacrificed and the bones were removed, imaged, fixed and then stained with hematoxylin and eosin (A) and with tissue Gram stain (B). No infiltrates or lesions of any kind were observed and the infected bones were indistinguishable from uninfected controls. (C) A bioluminescent derivative of *L. monocytogenes* strain EGDe also exhibits leg signals, apparently from the joint area in the mouse shown (a CD1 female on day 4). (D) The signal from the excised bones and joint of this animal emanated from the tip of the bone rather than from the joint itself (the BLI signal faded to reveal the tip of the bone). The uninfected [femur (E)] and infected [tibia (F)] bones were indistinguishable histologically and neither bone exhibited any evidence of infiltrate or granuloma. (G) Trichrome staining for bone integrity revealed no discernable degradation of bone material owing to infection and no differences from uninfected bone were evident (infected bone shown). Magnification: A, 50×; B,E,F, 100×; G, 200×.

orally infected mice can occur in different organs such as the gut, the liver or the gallbladder. The data presented in Fig. 1 demonstrates that the bone is also a site of re-emergence after oral inoculation. We have observed previously the re-emergence of gallbladder signals after as many as 15 days of undetectable levels (Hardy et al., 2004), indicating that the persistent bacteria were either dormant or below detectable numbers in the system we employed. Bone marrow might provide a location for dormant or small numbers of bacteria. Alternatively, the undetected bacteria could reside elsewhere and subsequently replicate when they somehow reach the bone marrow by normal circulation. Because detectable light emission by the *lux* system in *L. monocytogenes* requires bacterial replication, we cannot image dormant bacteria; however, the system is very sensitive for the detection of replicating bacteria, which, according to recoverable CFU, number only a few thousand in the foci that we observed. Thus, if the undetected bacteria were present in the bone marrow in small numbers of replicating cells, they must have been concentrated in groups of less than a few thousand. Intravenous infection produces intense, and often multiple, foci in the legs (Fig. 2). These signals appear at different days depending upon the dose employed and can occur at, or near, the joint as well as between the joints.

Relative to the number of CFU, the signals detected are much greater in the bone (3.1 photons per CFU per second) than in other organs such as the gallbladder (0.1 photons per CFU per second) – this is probably the result of the shallow depth of tissue in the leg relative to that of internal organs. Careful studies of the absorption of light by tissue in live animals (Zhao et al., 2005) have demonstrated that this property is highly dependent on not only tissue depth, but also the wavelength of light. The blue light emitted by the bacterial luciferase, which is emitted at a maximum wavelength (λ) of 490 nm, is more susceptible to attenuation by tissues than the green or red light of eukaryotic luciferases. Because the light emitted in the bone is very close to the surface of the animal, detection is greatly enhanced in comparison to other tissues and we easily observed foci that were composed of only a few thousand bacteria. This is also the case in the brain, where the infection can be close to the surface and imaging is very efficient (J.H. and C.H.C., unpublished). The imaging of excised organs can be useful, but it is not without limitations because, upon death, the transient loss of oxygen can cause severe signal reduction, and the signal may only return after excision of the organ and exposure to air (Wiles et al., 2006). In the case of the bones, excision and removal of tissue generated a signal that was 6-7 times greater than that from the live animal and demonstrated that the bacteria must still be metabolically active in the excised bone. The process of excision and imaging was, therefore, useful for the determination of signal location within the bone. In addition to the focus, some signals were distributed somewhat in the rest of the bone. In Fig. 3D (the bone on the right), the signal appears to be stronger close to the focus, which may indicate that the focus was spreading. Although we have observed signals emanating from the limbs in many animals, the only site in the limbs that we have confirmed harboring the bacteria has been the bone marrow. Lesions that appeared to be in the joints of intact animals were, following dissection and imaging of the excised bones, proven to be localized to the bone.

Perhaps the most unexpected finding from these studies was the persistence of highly attenuated mutants in the bone marrow for several weeks. These mutants are readily cleared from the liver, spleen and other organs of the body (Portnoy et al., 1988); however, they can persist in the lumen of the gallbladder, growing extracellularly to high numbers in this compartment (Hardy et al., 2004; Hardy et al., 2006). The gallbladder lumen is devoid of host cells and contains concentrated bile upon which the bacteria presumably feed. Therefore, this site is immunologically

privileged, at least with regard to cellular immunity, and attenuated bacteria are free to replicate there despite profound defects in intracellular growth. This is not the case in the bone marrow, which contains many cells, and how these highly attenuated mutants replicate in this location is mysterious. The increased persistence of attenuated mutants compared with wild-type bacteria, which, apparently, do not persist for more than 5 or 6 days, suggests that the lack of immune responses generated by the attenuated strains might play a role. Attenuated mutants probably remain within $CD31^+$, $Ly\text{-}6C^+$, $CD11b^+$ myeloid cells, as this location could provide protection from destruction by neutrophils and other phagocytic cells of the bone marrow. Perhaps, with the *hly* mutants, there is some residual escape from the vacuole owing to the broad range phospholipase (Marquis et al., 1995), which might permit replication in certain cells but not others. The presence of a signal indicates that the attenuated bacteria are growing and not merely present in a dormant state. The lack of a clearing immune response in the bone marrow is distinct from other sites that did not exhibit chronic foci. This distinction was most apparent with the attenuated strains, which persisted for many days in the bone marrow but not in other locations within the body.

The persistence of *L. monocytogenes* in the bone marrow has implications for human disease and treatment. In humans, infection of the bone and joints by *L. monocytogenes* is documented in the literature (Khan et al., 2001; Louthrenoo and Schumacher, 1990; Massarotti and Dinerman, 1990). In these cases the infection was severe enough to cause significant distress to the patient, such that a physician was consulted. Here, we demonstrate that apparently completely normal bones, in which no disease or destruction can be discerned by microscopy, can harbor infection. *L. monocytogenes* is currently a candidate for anti-cancer vaccines (Brockstedt et al., 2004; Yoshimura et al., 2006). Advaxis Inc. (New Brunswick, NJ) has reported encouraging Phase I clinical trial results regarding Lovaxin C, an anti-cervical cancer vaccine based on *L. monocytogenes*. All phases of infection by this bacterium must, therefore, be understood, particularly those associated with asymptomatic carriage or chronic maintenance. It is known that bacteria traffic to sites of inflammation; perhaps the sites of listerial bone and joint infection in patients were initially inflamed and then subsequently became infected. In the images shown here, although the hind limbs were infected, the front limbs were only rarely affected. Perhaps the mice were exhibiting low-grade stress to the hind limbs owing to the repetitive motion of rearing on their hind legs for food and water. This motion would not be expected to be repetitive in wild mice and, therefore, might stress the hind limbs of laboratory mice. If inflamed sites are more likely to be colonized, patients treated for cancer with recombinant *L. monocytogenes* should pay attention to any inflamed joints and report pain in the joints or bones. Bone marrow has also been shown to be the source of central nervous system infection in mice, and it is imperative to characterize the nature of listerial growth in this compartment with respect to listerial anti-cancer vaccines. Molecular imaging techniques that permit the analysis of small numbers of this bacterium, non-invasively, over time in live animals might also continue to reveal other unsuspected infection sites, improving our understanding of the host-pathogen relationship.

METHODS

Bacterial strains

Construction of the bioluminescent *L. monocytogenes* strain 2C, and the *hly*, *inlA*, *inlB* and *prfA* deletions of this strain from the parental *L. monocytogenes* strain 10403S, have been described previously (Hardy et al., 2004; Hardy et al., 2006). Briefly, *L. monocytogenes* strain 10403S (a gift from D. Portnoy) was made bioluminescent using the *lux-kan* hybrid transposon of the plasmid pAUL-A Tn4001 *luxABCDE* Km^r (Francis et al., 2001). Bacteria were initially selected using erythromycin, and subsequent insertions of the promoter-less hybrid *lux-kan* operon into listerial genes were selected using 200 μg/ml kanamycin. The insertions were sequenced by inverse PCR and characterized for light production and stability. The insertion of clone 2C was found to lie 47 base pairs downstream of the start codon of the *flaA* locus. The *flaA* gene is dispensable for virulence in *L. monocytogenes* strain 10403S (Way et al., 2004). This is a well-characterized locus with regard to expression (Grundling et al., 2004), so this very bright insertion was chosen as the basis for the comparison of mutants. The 2C strain was, therefore, used as a donor to transduce the *lux/kan* insertion into mutant backgrounds such as *Δhly*, *ΔinlA*, *ΔinlB* and *ΔprfA* of 10403S (also kindly provided by D. Portnoy). Transduction was performed according to Lauer et al. (Lauer et al., 2002) using the generalized transducing phage U153. Briefly, U153 lysates were prepared from the donor strain by incubating 300 μl of broth culture containing Luria Broth (LB), which had been aerated overnight, 10 mM $MgSO_4$ and 10 mM $CaCl_2$, with 10^3-10^4 plaque forming units (PFU) of U153, which was then plated in 3 ml of LB, with 0.7% agar overlays, in 10 cm LB agar petri dishes. Plates with interlaced plaque patterns were scraped, centrifuged and filtered for a donor stock, usually generating a titer of 2×10^8 PFU/ml. 200 μl of logarithmic recipient strain grown to an optical density of 0.2 was incubated with 50 μl or 100 μl of donor stock in brain heart infusion (BHI) broth containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$ for 1 hour, and then plated onto BHI 50 μg/ml kanamycin agar plates. The colonies were screened for bioluminescence using the IVIS100 and IVIS50 systems as per instruction of the manufacturer (Xenogen Corp., Alameda, CA – now part of Caliper Life Sciences). The bioluminescent derivative of *L. monocytogenes* strain EGDe was kindly provided by C. Gahan (University College Cork, Ireland). This strain contains one copy of a modified *luxABCDE* operon integrated into the chromosome, driven by the *hly* promoter (Corr et al., 2007).

Infection and imaging

Female BALB/c and CD1 outbred mice, aged 8-20 weeks, were injected intravenously with 200 μl of PBS containing $2\text{-}4\times10^4$ CFU of the virulent *L. monocytogenes* 2C or EGDe::pPL2 *luxABCDE* strains, or $1\text{-}2\times10^9$ CFU of the attenuated 2C *Δhly* or 2C *ΔprfA* strains or the 2C *ΔinlA*, *ΔinlB*, *Δhly* strain. The *hly* and *prfA* deletion strains behave identically with respect to BLI signal and exhibit a high degree of attenuation, each with an i.v. LD_{50} of approximately 2×10^9 CFU in 8-week-old female BALB/c mice. Mice were imaged each day after infection, as described below. The day of inoculation was designated day 0. After infection with 1×10^9 CFU of the attenuated strains, the mice often show symptoms of listeriosis, such as conjunctivitis of the eye,

lethargy, hunched appearance and matted fur, at day 1 post-inoculation. On day 2 or 3, the conjunctivitis is most often resolved and the animals are no longer lethargic or hunched in appearance. The mice generally recover from infection with the attenuated strains after 2 days and display no conjunctivitis or other symptoms thereafter. For oral infection, 2-5×10^9 CFU of *L. monocytogenes* strain 2C was administered to each mouse by mixing the inoculum with reconstituted, boiled nonfat milk and then feeding 20 µl to each mouse with a micropipette. The animals were then imaged 30 minutes after inoculation to detect the bacteria in the intestine. In the present study, the mice were imaged at each day post-inoculation, although they can be imaged several times in one day if necessary. Images were obtained using IVIS 100 and 200 systems (Xenogen Corp.) as per instruction of the manufacturer. Isoflurane gas anesthetic was administered at 2% in oxygen using the Matrix system (Xenogen Corp.), which enables the mice to recover from mild anesthesia within 2 minutes of the removal of gas. The images were obtained using an integration time of 3-5 minutes at a binning of 10×10 pixels for live animals, and a binning of 10×10 and 2×2 pixels for excised bones. Data analysis was performed using Living Image version 2.5 software and the IVIS system as per instruction of the manufacturer.

Ex vivo imaging and microscopy

Mice with leg signals were sacrificed on chosen days and the tibias or knee joints were dissected and imaged. Of the mice receiving attenuated strains at i.v. doses of 1-2×10^9 CFU, at least one or two out of each group of five animals displayed the femur signal. To obtain bioluminescence data from excised infected bones, the anesthetized mice were sacrificed when the desired signals were observed, and the femurs were then dissected and imaged in 10 cm petri dishes. It must be noted that images of excised tissues and organs may not be linear with respect to bacterial cell number, probably because oxygen saturation wanes after sacrifice (luciferase requires oxygen) and it cannot be assumed to be restored equally in all parts of all organs once they are excised and exposed to air. Nevertheless, signals from excised tissues and organs definitively indicate the presence of growing bacteria, and imaging excised infected tissues can be used to more accurately locate the signals that were observed in the live animal before sacrifice. Signals can be more accurately located in excised tissue compared with the intact animal because overlaying tissue scatters and absorbs light, and so removal of the tissue can increase the signal and improve resolution. The bones were imaged ex vivo with an IVIS 100 or IVIS 200, as described previously, using integration times of 5 minutes and a binning of either 2×2 or 10×10 pixels. Imaged bones were either processed for CFU determination or fixed and decalcified with a solution containing 1 M formaldehyde (Fisher). The fixed bones were sectioned and then stained with hematoxylin and eosin and a tissue Gram stain (Cullin, 1957), as well as with trichrome. For CFU determination, bone marrow was flushed from unfixed bones using a 20-gauge needle and homogenized in 0.1% Non-Idet P40 detergent and 10 mM phosphate, pH 7.4. The homogenate was then diluted and plated on BHI agar 60 µg/ml kanamycin plates and, 2 days later, colonies were verified as bioluminescent using an IVIS-50 system, as per the manufacturer's instructions.

TRANSLATIONAL IMPACT

Clinical issue

The pathogenic Gram-positive bacterium *Listeria monocytogenes* causes severe acute infection in immunocompromised people including the very young and the very old. *L. monocytogenes* is the third most common cause of bacterial meningitis in neonates and causes abortion and stillbirth. Outbreaks of listeriosis continue to occur sporadically, recently claiming 20 lives in Canada. Whereas acute listeriosis is well-modeled in animals, chronic infection is more difficult to study and is consequently poorly understood. Bone marrow has been shown to harbor *L. monocytogenes*, suggesting that bone might be an important chronic reservoir from which the pathogen can infect the central nervous system. However, this infection is difficult to study because it involves only a few bacteria and the extent of infection cannot be assessed until after the animal is sacrificed.

Results

Here, the authors use live animal imaging to examine bone marrow infection by *L. monocytogenes*. As in previous imaging studies of this pathogen in the gallbladder, they found that bone marrow is another site of chronic infection. In vivo bioluminescence demonstrated that bone marrow infection by this bacterium is focal, and that the foci appear after both oral and intravenous infection. Furthermore, although *L. monocytogenes* is hypothesized to be an obligate intracellular pathogen, highly attenuated mutants that are defective in intracellular replication can colonize the bone marrow, focally, for many weeks. Persistence of *L. monocytogenes* in this compartment supports the idea that the bone marrow is a niche for this, and perhaps other, pathogens.

Implications and future directions

Listeriosis can be very difficult to treat, sometimes requiring intravenous antibiotics for weeks. Studies of persistent bacterial infection should consider the bone marrow as a possible site of residual infection during, and after, treatment. In addition, this study demonstrates that the growth mechanism of attenuated *L. monocytogenes* is unclear; this is significant because attenuated strains are now in clinical trials to induce immune responses and eradicate established tumors without causing disease. The presence of *L. monocytogenes* in bone marrow might facilitate the induction of such immunity or, conversely, such persistence may indicate a lack of immune response and the induction of tolerance. Thus, in order to maximize efficacy and to ensure the safety of this treatment, future experiments should address immune stimulation in the bone marrow because this substantially sized niche might be beneficial, or detrimental, to anti-cancer therapy and other efforts.

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COMPETING INTERESTS

Christopher Contag is a founder of Xenogen Corporaiton – now Caliper Life Sciences.

AUTHOR CONTRIBUTIONS

J.H. infected, imaged and dissected animals. P.C. performed histological analysis. J.H., P.C. and C.H.C. conceived experiments and analyzed data.

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