Chapter 3
Caenorhabditis elegans as an Alternative Model to Study Senescence of Host Defense and the Prevention by Immunonutrition

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Abstract Whether nutritional control can retard senescence of immune function and decrease mortality from infectious diseases has not yet been established; the difficulty of establishing a model has made this a challenging topic to investigate. *Caenorhabditis elegans* has been extensively used as an experimental system for biological studies. Particularly for aging studies, the worm has the advantage of a short and reproducible life span. The organism has also been recognized as an alternative to mammalian models of infection with bacterial pathogens in this decade. Hence we have studied whether the worms could be a model host in the fields of immunosenescence and immunonutrition. Feeding nematodes lactic acid bacteria (LAB) resulted in increases in average life span of the nematodes compared to those fed *Escherichia coli* strain OP50, a standard food bacteria. The 7-day-old nematodes fed LAN from age 3 days were clearly endurable to subsequent salmonella infection compared with nematodes fed OP50 before the salmonella infection. The worm could be a unique model to study effects of food factors on longevity and host defense, so-called immunonutrition. Then we attempted to establish an immunosenescence model using *C. elegans*. We focused on the effects of worm age on the *Legionella* infection and the prevention by immunonutrition. No significant differences in survival were seen between 3-day-old worms fed OP50 and 3-day-old worms infected with virulent *Legionella* strains. However, when the worms were infected from 7.5 days after hatching, the virulent *Legionella* strains were obviously nematocidal for the worms' immunosenescence. In contrast, nematodes fed with bifidobacteria prior to *Legionella* infection were resistant to *Legionella. C. elegans* could act as a unique alternative host for immunosenescence and resultant opportunistic infection, and immunonutrition researches.

Introduction

Caenorhabditis elegans is a small free-living soil nematode that feeds on bacteria; it has been extensively used as an experimental system for biological studies because of its simplicity, transparency, ease of cultivation, and suitability for genetic analysis (Riddle et al. 1997). Particularly for aging studies,

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E. Mylonakis et al. (eds.), *Recent Advances on Model Hosts*,
Advances in Experimental Medicine and Biology 710, DOI 10.1007/978-1-4419-5638-5_3,

the worm has the advantage of a short and reproducible life span (Finch and Ruvkun 2001). Recently, after Ausubel et al. reported infection due to *Pseudomonas aeruginosa* (Kurz and Tan 2004; Nicholas and Hodgkin 2004; Tan et al. 1999), the organism has also been recognized as an alternative to mammalian models of infection with bacterial pathogens. In the field of innate immunity research, *C. elegans* is becoming one of the most important experimental animals, similar to the fruit fly *Drosophila* (Kurz and Tan 2004; Nicholas and Hodgkin 2004; Schulenburg et al. 2004).

Age at infection is one of the most important determinants of disease morbidity and mortality (Miller and Gay 1997). Because aging is accompanied by functional and metabolic alterations in cells and tissues, senescence of the immune system results in an age-related increase of infections, malignancy, and autoimmunity (Grubeck-Loebenstein 1997; Moulias et al. 1985). Elderly humans have increased mortality from many different types of infections (Bradley and Kauffman 1990).

Whether nutritional control can retard senescence of immune function and decrease mortality from infectious diseases has not yet been established; the difficulty of establishing a model has made this a challenging topic to investigate. Although some studies have shown successful improvement of biomarkers relating to immunological functions (Bogden and Louria 2004), few reports have shown a beneficial influence of nutrition on immunity and the resultant outcome of experimental infection (Hayek et al. 1997; Effros et al. 1991). Hence we have studied whether *C. elegans* is a useful model host in the fields of immunosenescence and immunonutrition.

C. elegans as a Model for Immunonutrition

Probiotic bacteria are defined as living microorganisms that exert beneficial effects on human health when ingested in sufficient numbers (Naidu et al. 1999). Lactic acid bacteria (LAB) have been used in various fermented foods since antiquity. Metchnikoff, who first proposed the concept of probiotic bacteria in 1907, hypothesized that lactobacilli were important for human health and longevity (Metchnikoff 1907). LAB are the most commonly used probiotic microorganisms. LAB have been found to have a variety of physiological influences on their hosts, including antimicrobial effects, microbial interference, supplementary effects of nutrition, anti-tumor effects, reduction of serum cholesterol and lipids, and immunomodulatory effects. However, there have been no reports concerning the influence of LAB on longevity and immunosenescence.

First, we evaluated whether LAB could contribute to host defenses and prolong the lifetime of *C. elegans* (Ikeda et al. 2007). Lactobacilli and bifidobacteria were fed to worms, and their life span and resistance to *Salmonella enterica* were compared with those of worms fed *Escherichia coli* OP50, an international standard food for *C. elegans*. The worms were generally infected with inocula on conventional nematode growth medium, which contains peptone, raising the possibility that the inoculated pathogen would have proliferated regardless of whether it could successfully infect the nematodes and derive nutrition from the hosts. Garsin et al. showed that nutrition available in agar plates does influence the virulence of pathogens on the media (Garsin et al. 2001). Furthermore, some pathogens produce toxic metabolites on nutrient medium in situ (Anyanful et al. 2005). To avoid such a condition, our experiments were performed on modified nematode growth medium (mNGM) containing no peptone as we reported before (Hoshino et al. 2008). Worms fed heat-killed OP50 reportedly live longer than those fed alive bacteria on nutrient NGM, however this difference was not observed on modified NGM.

Feeding nematodes bifidobacteria or lactobacilli resulted in increases in average life span of the nematodes compared to those fed OP50 (Fig. 3.1). To examine whether or not the beneficial effects of LAB were brought about by their harmless nature compared to OP50, survival was compared with that of nematodes fed on heat-killed OP50. Heat-killed OP50 did not prolong the worms' longevity as much as LAB did (Fig. 3.2).

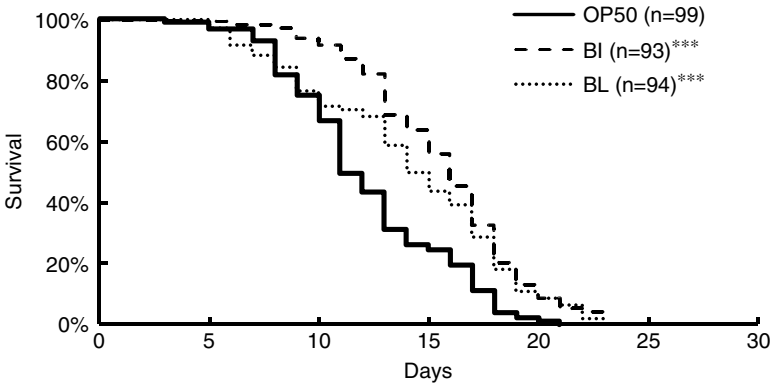

Fig. 3.1 Effects of lactic acid bacteria on the life span of *C. elegans*. Adult worms fed a diet of *E. coli* strain OP50 for 3 days after hatching were transferred to diets of bifidobacteria. The bifidobacteria used were *B. infantis* (BI) or *B. longum* (BL). The life spans of nematodes fed bifidobacteria were significantly extended (***$p<0.001$). The mean life spans (in days) of worms fed *B. infantis* or *B. longum* were 15.1 ± 0.40 (29%) and 13.6 ± 0.50 (17%), respectively: numbers in parentheses are percentage differences in the mean relative to controls fed OP50

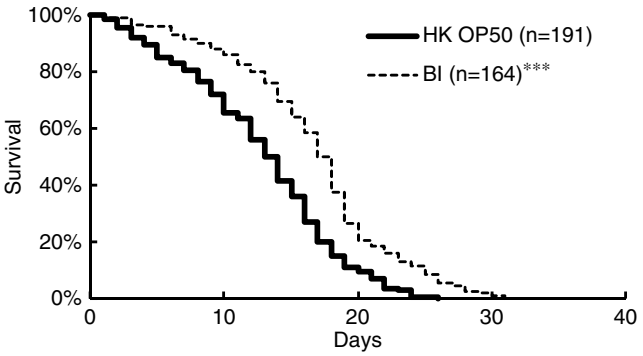

Fig. 3.2 The mean life span (16.3 ± 0.47 days) of worms fed *B. infantis* was prolonged by 33% over that seen with worms fed heat-killed OP50 (12.27 ± 0.42 days) (***$p<0.001$)

Salmonella killed about 40% of the nematodes in 5 days after the worms were transferred to the lawn of this pathogen at age 7 days, while 80% of the worms fed OP50 remained alive after 5 days. The 3-day-old worms were not killed in 5 days when fed either OP50 or *Salmonella*. The 3-day-old worms were clearly more resistant to *Salmonella* compared to the 7-day-old nematodes (Fig. 3.3); the initial number of *Salmonella* recovered from those worms in which infection started at age 3 days was smaller than the number recovered from worms infected from age 7 days (Fig. 3.4).

Importantly, 7-day-old nematodes fed bifidobacteria or lactobacilli from age 3 days were clearly more tolerant to subsequent *Salmonella* infection compared with nematodes fed OP50 before the *Salmonella* infection (Fig. 3.5). LAB seem to make the worms tolerant rather than resistant to *Salmonella* infection; the number of *Salmonella* recovered from worms fed LAB was the same as that recovered from worms grown on OP50.

The mechanisms how LAB brought the worms longevity effects and made them tolerant have not been elucidated. However, if the increased longevity was due to enhancement of host defenses

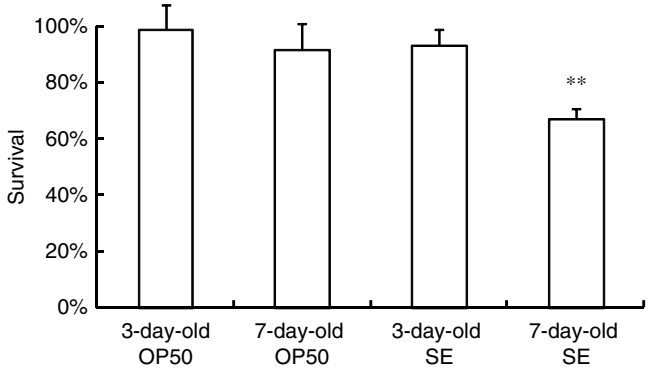

Fig. 3.3 Survival rate at the fifth day of *Salmonella enterica* serovar *Enteritidis* (SE) infection. The death rate of nematodes infected at age 7 days was greater than that of worms infected at age 3 days (**$p<0.01$). All results are presented as the mean ± standard error of the mean

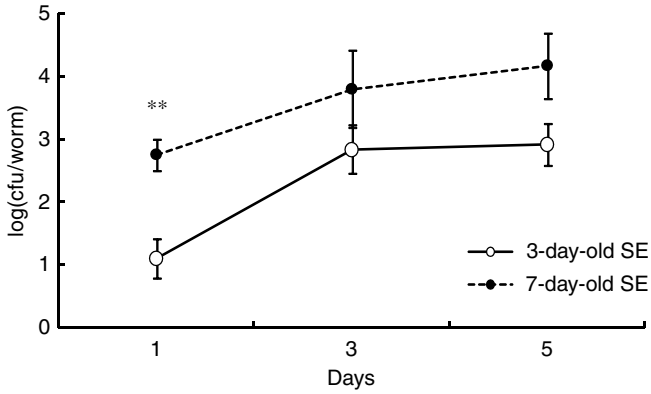

Fig. 3.4 The number of *Salmonella* recovered from young nematodes on the first day after the infection was significantly lower than the number recovered from worms infected at age 7 days (**$p<0.01$). All results are presented as the mean ± standard error of the mean

as one of the probiotic effects, the worm could be a unique model to study effects of not only LAB but other nutrients on host defense, so-called immunonutrition. *C. elegans* is useful for studying the relationship between innate immunity and pathogens because the nematode lacks an adaptive immune system. Although *C. elegans* does not have phagocytes specialized for innate host defense, it produces a variety of humoral antibiotic substances such as lysozymes, caenopores, lipase, lectins and C3-like thioester-containing proteins, and defensin-like antibiotic peptides. These substances in the bacteriophagous nematodes might be considered to be digestive enzymes; the worm's intestine could be considered analogous to a phagosome. Bacteria resistant to these antibacterial substances are more likely to be nematocidal. Consequently, *C. elegans* may be most suitable to study anti-innate immunity properties of pathogens since the organisms have to contend with the humoral defense factors produced by the host.

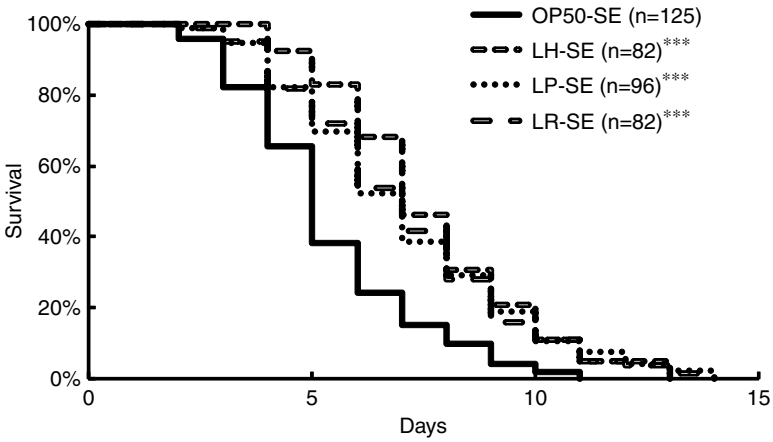

Fig. 3.5 Effects of lactic acid bacteria on resistance of *C. elegans* to *Salmonella*. Adult worms fed a diet of *E. coli* strain OP50 for 3 days after hatching were transferred to a diet of lactobacilli. The lactobacilli used were *L. helveticus* (LH), *L. plantarum* (LP), or *L. rhamnosus* (LR). Four days later the nematodes were transferred to *Salmonella* plates, and survival curves were determined. Nematodes fed each type of lactobacilli were significantly more resistant than controls to the pathogen (***$p < 0.001$). Mean days of survival of worms fed LH, LP, LR before the salmonella infection were 7.1 ± 0.25 (46%), 6.6 ± 0.28 (35%), and 6.6 ± 0.27 (35%), respectively; numbers in parentheses are percentage differences in the mean relative to controls fed OP50

C. elegans as a Model for Immunosenescence

In a second step, we attempted to establish an immunosenescence model using *C. elegans*. Although *Salmonella enterica* serovar Enteritidis killed old nematodes more quickly after the infection than young worms as we described above, we wanted to develop a model capable of testing whether opportunistic infections increase due to immunosenescence. *Legionella pneumophila*, an environmental bacterium naturally found in fresh water, is the major causative agent of Legionnaires' disease (Fields et al. 2002). Fresh water amoeba, a natural host of *Legionella*, has been used as an infection model to study invasion of *Legionella* into human macrophages and subsequent intracellular growth (Jules and Buchrieser 2007). However, analyses using these protozoa have inevitably concentrated on the intracellular lifestyle of *L. pneumophila*. The fate of *Legionella* organisms in non-mammalian metazoans had not been reported (Hilbi et al. 2007) until a very recent report by Brassinga et al. (Brassinga et al. 2010). Since *Legionella* is prone to infect elderly people, we focused on the effects of worm age on *Legionella* infection and the prevention of infection by immunonutrition (Komura et al. 2010). Infections in young and old nematodes were compared. Furthermore, survival curves were compared between worms fed with OP50 and those fed bifidobacteria prior to infection with *Legionella* organisms, since lactic acid bacteria exert beneficial effects on human and animal health (Naidu et al. 1999).

No significant differences in survival were seen between 3-day-old worms fed OP50 and 3-day-old worms infected with virulent *Legionella* strains. However, when the worms were infected from 7.5 days after hatching, the virulent *Legionella* strains were obviously nematocidal (Fig. 3.6). These data show that *L. pneumophila* is virulent even on peptone-free mNGM if the targets are elderly worms. Our previous study showed that *Salmonella* is clearly virulent to both older and younger worms, although more so in elderly worms (Ikeda et al. 2007). These findings appear to be similar to the epidemiological characteristics of both pathogens in humans: *Legionella* tends to infect older people in an opportunistic manner, while *Salmonella* can cause enteritis irrespective of host age.

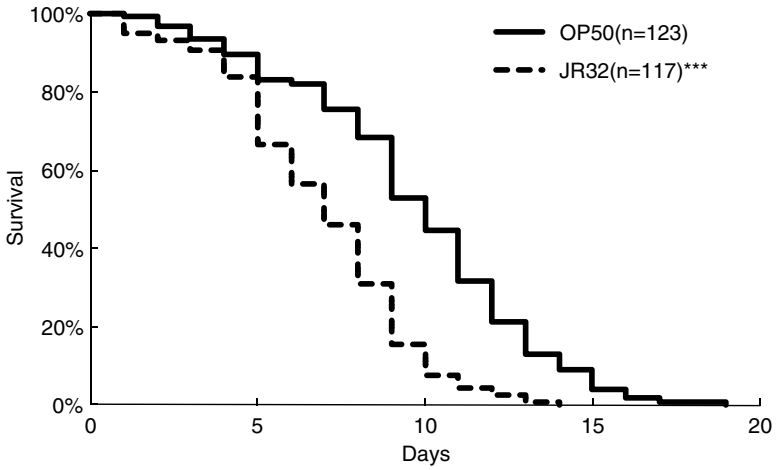

Fig. 3.6 Survival of nematodes infected with *L. pneumophila*. From 7.5 days of age, nematodes were transferred to agar plates covered with the *L. pneumophila* virulent strain JR32. The survival curves were compared with that of worms fed on OP50 (***p<0.001)

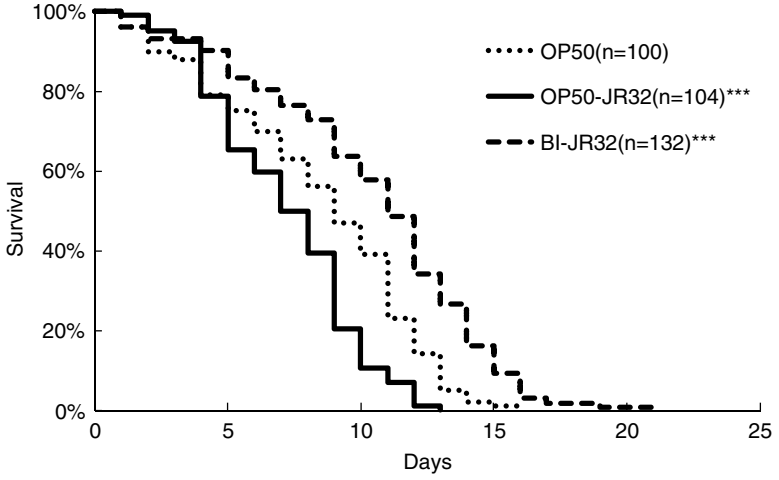

Fig. 3.7 Adult worms fed a diet of bifidobacteria from 3 days of age for 5 days were transferred to *Legionella* plates for infection, and survival curves were drawn. Nematodes fed bifidobacteria were significantly more tolerant than controls to the pathogen (***p<0.001)

As with the case of many other pathogens in the *C. elegans* model, *Legionella* mutants that are less virulent in the lungs of guinea pigs (Miyamoto et al. 2003) or in human macrophages (Sadosky et al. 1993), are also less virulent in *C. elegans*. Interestingly, the attenuated mutant LELA 1718, which is reportedly cytolethal compared to the other attenuated mutants in a cytotoxicity assay with HL-60-derived human macrophages (Sadosky et al. 1993), showed modest virulence in the nematode compared to other avirulent mutants. The pathogenicity of *L. pneumophila* in *C. elegans* seems to correlate well with that in macrophages, and the nematode could serve as a unique host of *Legionella* spp.

Nematodes fed with bifidobacteria prior to *Legionella* infection were resistant to *Legionella* (Fig. 3.7). The number of *Legionella* recovered from the worms showed no significant difference

between groups fed with bifidobacteria or OP50. This phenomenon is similar to the tolerance that we observed when nematodes were fed lactic acid bacteria prior to *Salmonella* infection (Ikeda et al. 2007).

Development of a Method for Oral Administration to Nematodes

Despite increased use of *C. elegans* in a variety of studies, there is no efficient method to administer chemicals orally. When chemicals need to be administered to nematodes, they are either dissolved in the NGM or the solution is poured onto the OP50 lawn. *C. elegans* ingests relatively large particles such as bacteria, that are suspended in water, and then spits out much of the liquid, while retaining the particles (Avery and Thomas 1997). This feeding behavior is likely to be inefficient for ingestion of solutions.

In the third step of our studies, we aimed to develop methods for oral administration that is essential for developing a biologically relevant *C. elegans* immunonutrition model. We hypothesized that nematodes would be able to take up liposomes, similar to their ingestion of bacteria. We used liposomes loaded with the hydrophilic fluorescent reagent uranin to test oral administration of water-soluble substances to *C. elegans*, and compared the efficiency of liposome-mediated delivery with conventional methods (Shibamura et al. 2009).

Dietary supplements of antioxidants were previously reported to have positive effects on longevity, while other studies reported controversial results. Water-soluble antioxidants were administered using both our newly developed liposome method and conventional methods to compare the effect on lifespan of nematodes and on host defense against *Salmonella* infection. Using our the liposome method, we showed marked longevity effects of antioxidants on the lifespan of *C. elegans* (Fig. 3.8). Oral administration was more than 200 times as efficient as the conventional method in dose response tests. We expect that this method could open new phase of *C. elegans* research as a model host.

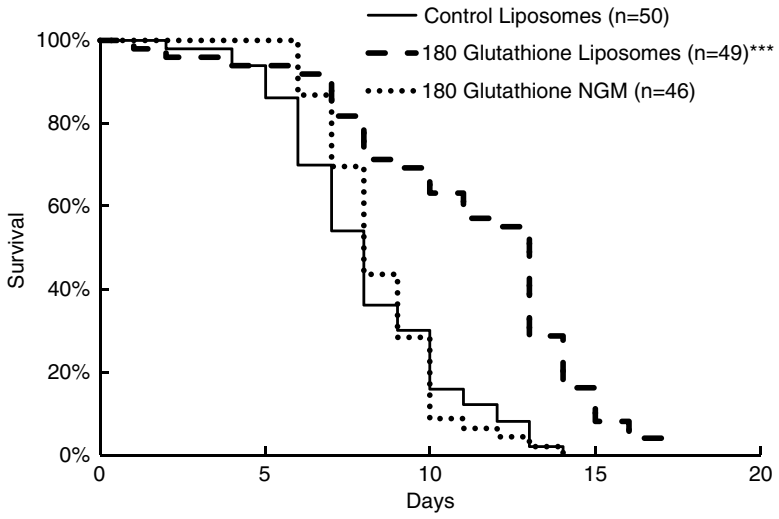

Fig. 3.8 Survival curves of nematodes supplemented with 25 μL of liposomes containing 180 μg reduced glutathione. After hatching, nematodes were grown on *E. coli* OP50 for 3 days, and then the adult worms were divided into groups that were supplemented with chemicals. Water-containing liposomes were administered to control worms and those maintained on mNGM containing 180 μg of glutathione. ***$p < 0.001$, compared to the control

Conclusion

Due to increasing ethical considerations as well as economic reasons, the use of mammalian hosts is decreasing in popularity. We showed that *C. elegans* could act as a unique alternative host for immunosenescence and resultant opportunistic infection and immunonutrition experiments. Compared with murine infection models, it is not easy to extrapolate whether the nematocidal activity of a particular pathogen would be reflected in virulence in human pathogenesis. However, for simplicity, transparency, ease of cultivation, and suitability for genetic analysis, *C. elegans* is a uniquely useful model. Particularly for studies on aging of host defense, the worm has the great advantage of a short and reproducible life span.

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