Programmed cell death mediated by *ced-3* and *ced-4* protects *Caenorhabditis elegans* from *Salmonella typhimurium*-mediated killing

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Programmed cell death (PCD) in mammals has been implicated in several disease states including cancer, autoimmune disease, and neurodegenerative disease. In *Caenorhabditis elegans*, PCD is a normal component of development. We find that *Salmonella typhimurium* colonization of the *C. elegans* intestine leads to an increased level of cell death in the worm gonad. *S. typhimurium*-mediated germ-line cell death is not observed in *C. elegans ced-3* and *ced-4* mutants in which developmentally regulated cell death is blocked, and *ced-3* and *ced-4* mutants are hypersensitive to *S. typhimurium*-mediated killing. These results suggest that PCD may be involved in the *C. elegans* defense response to pathogen attack.

Several human pathogens, including *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, and *Burkholderia pseudomallei*, kill the nematode *Caenorhabditis elegans* when supplied as a food source, and a variety of bacterial virulence factors have been shown to play a role in both nematode and mammalian pathogenesis (1–6). In addition, *C. elegans* mutants that are either more susceptible or more resistant to bacterial killing can be readily identified (7, 8). These facts make *C. elegans* an attractive host for dissecting the molecular basis of bacterial pathogenesis.

An important feature of a variety of mammalian–pathogen interactions is programmed cell death (PCD) of both immune and somatic cells. PCD in mammals has also been implicated in several disease states, including cancer, autoimmune disease, and neurodegenerative disease (9). Because many of the key components of the mammalian cell death machinery were first identified by genetic studies in *C. elegans* (10, 11), it was of interest to determine the role, if any, of PCD in *C. elegans* in response to pathogen attack.

Mutations in at least three *C. elegans* genes, *ced-3*, *ced-4*, and *ced-9*, affect the 131 somatic cell deaths that occur during the development of the *C. elegans* hermaphrodite (12, 13). Loss-of-function mutations in *ced-3* or *ced-4* or a gain-of-function mutation in the gene *ced-9* result in the survival of cells that normally die. CED-3 and CED-4 proapoptotic activity is antagonized by the Bcl-2 family member CED-9 (14). In cells fated to die, EGL-1 binds to and directly inhibits the activity of CED-9 (15).

The 131 cells that undergo PCD during the somatic development of *C. elegans* represent about 12% of the cells in an adult worm. In contrast to somatic cells, germ cells do not have a fixed lineage or population of cells. In normal adult hermaphrodites, over half of all potential oocytes are eliminated by PCD that is mediated by the same core machinery (*ced-3*, *ced-4*, and *ced-9*) responsible for somatic PCD during development (16). Germ cell deaths are more abundant in starving and old worms than in well fed young animals (17), suggesting that PCD in the germ line is regulated by environmental conditions.

P. aeruginosa and *S. typhimurium* kill *C. elegans* by at least three distinct mechanisms when provided to *C. elegans* as the sole source of food. At least in the case of *P. aeruginosa*, the mechanism of killing depends on the strain of *P. aeruginosa* used and the medium used to grow the bacteria. Typically, *C. elegans* are propagated in the laboratory by feeding them on *Escherichia coli* strain OP50 grown on NG medium. When fed on *P. aeruginosa* strain PA14 grown on the relatively low osmolarity NG medium, PA14 accumulates within the lumen of the *C. elegans* intestine, killing worms relatively slowly over the course of two–three days (slow killing; ref. 5). In contrast, PA14 grown in a rich and high osmolarity medium kills worms more quickly through a toxin-mediated mechanism (fast killing; refs. 5 and 8). *P. aeruginosa* strain PAO1 grown on brain-heart infusion medium kills by a third mechanism involving the generation of one or more neurotoxins (7). A variety of *S. enterica* serovars, including *S. typhimurium*, grown on NG medium, kill *C. elegans* over the course of several days by a mechanism that involves the establishment of a persistent infection in the *C. elegans* intestine (1).

We tested whether germ-line cell death plays a role in the *C. elegans* defense response to pathogen attack by examining the interaction between *P. aeruginosa* or *S. typhimurium* and *C. elegans* mutants deficient in PCD. Our results show that *S. typhimurium* elicits germ-line cell death, and that *C. elegans ced-3* and *ced-4* mutants are more susceptible to *S. typhimurium*-mediated killing. In contrast, germ-line cell death does not seem to play a significant role in the *C. elegans*—*P. aeruginosa* interaction.

Materials and Methods

Bacterial Strains and Growth Conditions. *E. coli* OP50 (18), *S. typhimurium* SL1344 (19), *S. typhimurium* 14028 (20), or *S. typhimurium* LH954 (20) were grown overnight at 37°C in Luria–Bertani medium. Bacterial lawns used for *C. elegans* killing assays were prepared by spreading 10 μl of the bacterial strains on modified nematode growth agar medium (0.35% instead of 0.25% peptone) in 3.5-cm diameter plates. Plates were incubated at 37°C for 12 h and then allowed to equilibrate to room temperature for 3 h before seeding them with worms.

***C. elegans* Killing Assay.** Ten worms were placed on a bacterial lawn and the plates were incubated at 20°C. Each independent assay was carried out in duplicate. During the reproductive period, adults were transferred daily to fresh plates and, thereafter, were transferred approximately every 5 days. Worm mortality was scored over time, and a worm was considered dead when it failed to respond to touch. The time for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated by using the PRISM V. 2.00 computer program using the equation:

Abbreviations: PCD, programmed cell death; TD_{50}, time to death for 50% of worms.

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Y = Bottom + (Top − Bottom)/(1 + $10^{(\log EC_{50} - X)\cdot \text{Hill slope}}$), where X is the logarithm of days and Y is the average of dead worms. The relative mortality of worms feeding on *S. typhimurium* was calculated with the equation: Relative mortality = (TD_{50} wild-type *C. elegans* on *S. typhimurium*/TD_{50} *C. elegans* mutant on *S. typhimurium*)/(TD_{50} wild-type *C. elegans* on *E. coli*/TD_{50} *C. elegans* mutant on *E. coli*).

Nematode Strains. All *C. elegans* strains were maintained as hermaphrodites at 20°C, grown on modified NG agar plates (0.35% peptone instead of 0.25%) and fed with *E. coli* strain OP50 as described (18). The *C. elegans* mutants used were derived from the wild-type variety Bristol N2. *ced-3(n717)* and *ced-9(n1653ts)* mutants were kindly provided by Michael Hengartner, Cold Spring Harbor Laboratory. *ced-3(n1286)*, *ced-3(n2433)*, *ced-4(n1162)*, *ced-4(n1894)*, *egl-1(n1084n3082)*, *ces-1(n703)*, and *ces-2(n732)* were obtained from the *Caenorhabditis* Genetics Center, University of Minnesota, St. Paul.

Cell Corpse Assay. To quantify the number of apoptotic germ cells, the animals were stained with SYTO 12 (Molecular Probes) as previously described (16). Briefly, the worms were incubated in 50 μM SYTO 12 for 3–4 h at room temperature and then seeded on bacterial lawns to reduce the amount of stained bacteria in the gut. After 20–30 min, animals were mounted in a drop of M9 salt solution containing 30 mM NaN_3 and observed by using a Leica TCS SP confocal microscope. Only animals that were brightly and equally stained were scored.

Results

***S. typhimurium* but Not *P. aeruginosa* Elicits Germ-Line Cell Death.** Both L4 and one-day-old *C. elegans* adults died more quickly when fed on *S. typhimurium* strain SL1344 than when fed on *E. coli* strain OP50, the usual food source for growing *C. elegans* in the laboratory. Because we also observed that *S. typhimurium* infection results in an approximately 30% ± 10% decrease in brood size (data not shown), we determined whether *S. typhimurium* affects the rate of germ-line cell death by identifying germ-line cell apoptotic corpses with Nomarski optics or by staining with the nucleic acid stain SYTO 12 as described in *Materials and Methods*. SYTO 12 specifically stains condensed structures in the gonad of adult hermaphrodites that are apparently the corpses of apoptotic germ-line cells that have undergone PCD (16).

A higher rate of apoptosis was observed in *C. elegans* germ cells when the worms were fed *S. typhimurium* SL1344 than when fed *E. coli* OP50 (Fig. 1*A* and Table 1). The higher percentage of apoptotic corpses in infected worms persisted throughout the reproductive life of the animals. The condensed structures identified as apoptotic corpses observed in worms feeding on *E. coli* (Fig. 1*B*) or *S. typhimurium* (Fig. 1*D*) were located in a region of the germ line occupied by syncytial germ cells undergoing meiosis and corresponded to the structures stained by SYTO 12 (Fig. 1 *E* and *C*).

To evaluate whether the mere presence of *S. typhimurium* in the *C. elegans* intestine is capable of inducing PCD or whether PCD requires the expression of specific virulence factors, we analyzed the role of the *S. typhimurium* PhoP/PhoQ signal transduction system, a key regulator of virulence-related genes (21). We took advantage of an *S. typhimurium* mutant (LH 954) with a *phoP/phoQ/purB* deletion that we previously showed causes significantly less killing of *C. elegans* (1). The results shown in Table 1 indicate that the *phoP/phoQ/purB* (LH 954) mutant kills *C. elegans* at a slower rate than the parental *S. typhimurium* strain (SL14028) and does not elicit an apoptotic response.

In contrast to *S. typhimurium*, we did not observe an elevated rate of germ-line cell death when *C. elegans* was fed *P. aeruginosa* strain PA14 under either the so-called slow (Table 1) or fast (data not shown) killing conditions. Surprisingly, only approximately 5% of the worms that had been in contact with *P. aeruginosa* for 3 h under the slow killing conditions were capable of taking up the SYTO 12 stain. This latter result suggests that despite the lipidic nature of the dye, some kind of active transport may be required for the uptake of the dye, and that *P. aeruginosa* blocks this process.

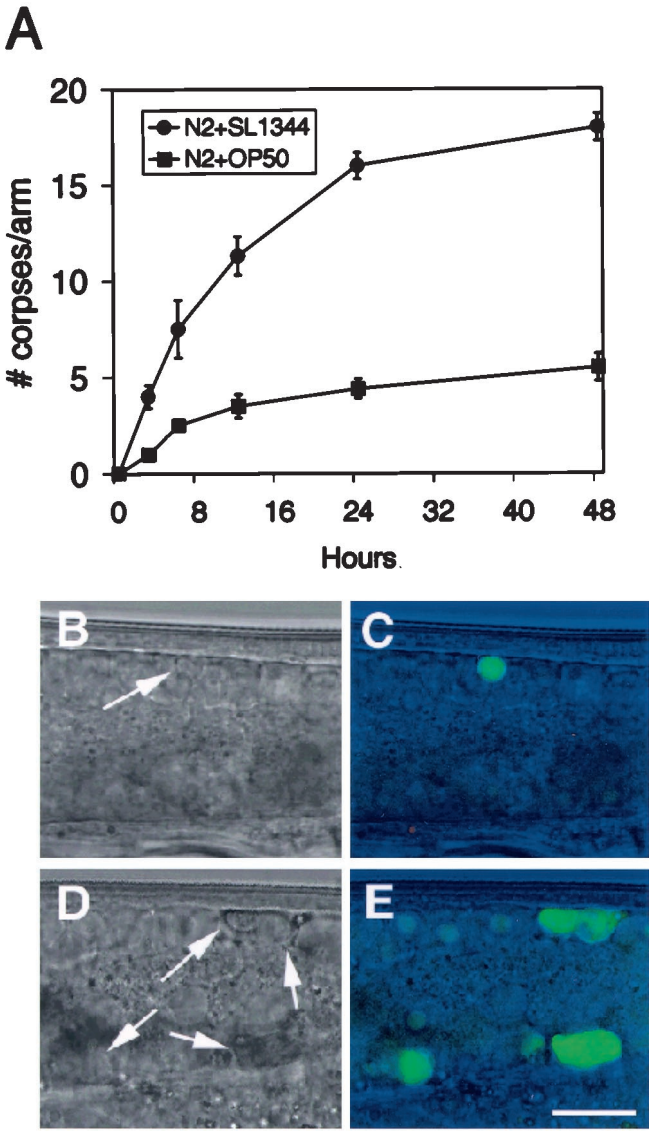

Fig. 1. *S. typhimurium* induces PCD in the *C. elegans* germ line. (*A*) Cell corpses were counted over time in one gonad arm, starting with young adult animals fed on *E. coli* OP50 or *S. typhimurium* SL1344. Data (mean ± SD) are from at least three independent experiments; more than 15 animals were scored at each time point. (*B–E*) Confocal images showing 1-day-old hermaphrodite worms fed on *E. coli* OP50 (*B* and *C*) or *S. typhimurium* SL1344 (*D* and *E*) for 12 h. In the transmission images (*B* and *D*), the cell corpses are indicated with arrows. Merged images (*C* and *E*) show corpses stained with SYTO 12. (Bar = 30 μm.)

***S. typhimurium*-Induced Germ-Line Cell Death Depends on the *ced-3/ced-4* Cell Death Pathway.** We used a variety of *C. elegans* mutants that affect PCD in somatic and germ-line cells to determine whether the *S. typhimurium*-induced apoptosis in the germ line requires previously identified apoptotic machinery and whether this

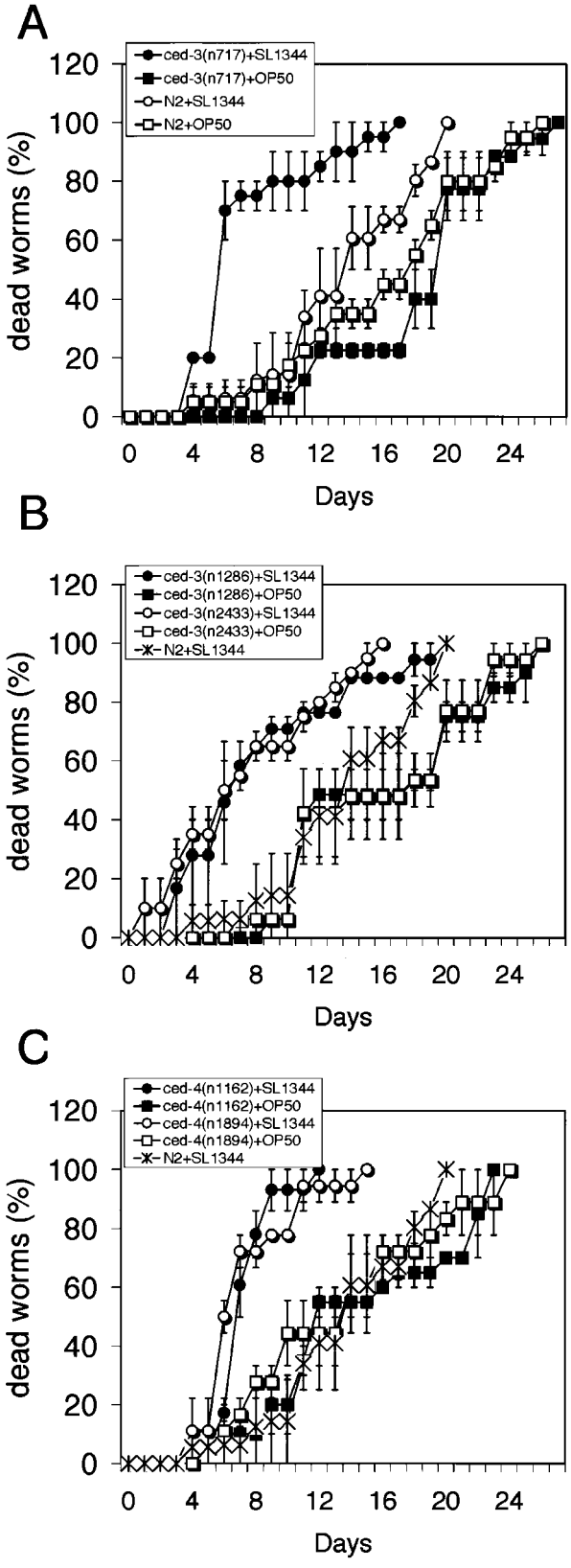

Fig. 2. *ced-3* and *ced-4* mutants are more susceptible to *S. typhimurium*-mediated killing. One-day-old adult animals were fed either *E. coli* or *S. typhimurium*. (*A*) Wild type and *ced-3(n717)*. (*B*) Wild type, *ced-3(n1286)*, and *ced-3(n2433)*. (*C*) Wild type, *ced-4(n1162)*, and *ced-4(n1894)*. More than 10 animals were used in each case and data (mean ± SD) were from duplicates. The results are representative of at least three experiments.

apoptotic machinery is involved in *S. typhimurium*-mediated killing. The proximal cause of apoptosis in *C. elegans* is the activation of CED-3, which is required for germ cell death (16). As shown in Table 1, no germ-line cell deaths were observed in a *ced-3* mutant feeding on *E. coli* or *S. typhimurium. ced-3* encodes a prototypical caspase (22), and CED-4 is similar to mammalian Apaf-1, an activator of caspases (23) that binds and activates CED-3 (24–26). Thus, if activated CED-3 is required for *S. typhimurium*-induced cell death, *ced-4* mutants should also impair cell death in *Salmonella*-infected worms. As observed in *ced-3* animals, no germ-line cell deaths were observed in *ced-4* mutants feeding on *S. typhimurium* lawns (Table 1).

***C. elegans ced-3* and *ced-4* Mutants Are Hypersusceptible to *S. typhimurium*-Mediated Killing.** As shown in Fig. 2 *A* and *B*, *ced-3* mutants died much more quickly than wild-type worms when feeding on *S. typhimurium*, but died at the same rate as wild-type worms when feeding on *E. coli*. The TD_{50} for 1-day-old hermaphrodite nematodes when fed at 20°C on *S. typhimurium* was 14 days for wild-type worms compared with 5.9, 6.6, and 6.5 days (respectively) for three different *ced-3* mutant alleles, whereas the TD_{50} for the three *ced-3* mutants feeding on *E. coli* was 19, 16, and 16 days (respectively) compared with 17 days for wild-type worms feeding on *E. coli*. In addition, the rate at which wild-type worms died when feeding on *S. typhimurium* (TD_{50} = 14 days) was faster than the rate at which both wild-type worms (TD_{50} = 17) and *ced-3* mutant worms (TD_{50} = 19, 16, 16 days) died when feeding on *E. coli* (for example, see Fig. 2*A*). Two different *ced-4* mutants were also more susceptible to *S. typhimurium*-mediated killing (Fig. 2*C*). The fact that three *ced-3* alleles and two *ced-4* alleles exhibited the same phenotype when feeding on *S. typhimurium* makes it unlikely that enhanced susceptibility is caused by secondary mutations or the effect of a particular allele on a process unrelated to cell death. Consistent with the observation that *P. aeruginosa* strain does not induce a high level of germ-line PCD, *ced-3* and *ced-4* mutants were not more susceptible to *P. aeruginosa*-mediated fast or slow killing (data not shown). These experiments, therefore, are consistent with the conclusion that CED-3 and CED-4 are involved in a *C. elegans* defense response to *S. typhimurium* but not to *P. aeruginosa*.

The *C. elegans* Upstream Regulators of Cell Death CED-9 and EGL-1 Are Also Involved in *S. typhimurium*-Induced Germ-Line Cell Death. CED-9 is a member of the Bcl-2 family of cell death regulators (14) that directly inhibits CED-4, apparently by sequestering CED-4 and proCED-3 in an inactive ternary complex called the apoptosome (27). The *ced-9(1950)* gain-of-function mutant has a similar phenotype to *ced-3* and *ced-4* loss-of-function mutants with respect to PCD in developing worms (14). EGL-1 is thought to activate the PCD cascade by inhibiting CED-9 (15). Consistent with the results described in the previous section, Table 1 shows that *Salmonella*-induced apoptosis is substantially reduced in the *ced-9(n1950)* mutant. Similarly, the *egl-1(n1084n3082)* loss-of-function mutant also exhibited a reduction in *Salmonella*-induced germ-line apoptosis compared with wild-type N2 worms (Table 1). Moreover, both the *ced-9(n1950)* and the *egl-1(n1084n3082)* mutants were more susceptible to *Salmonella*-mediated killing (Fig. 3 *A* and *C*).

Consistent with previous observations that *ced-9* loss-of-function mutants exhibit a much higher rate of spontaneous cell death than wild-type worms (16), the *ced-9(n1653ts)* loss-of-function mutant exhibited elevated PCD in the gonads (data not shown). Fig. 3*B* shows that the *ced-9(n1653ts)* mutant had a short lifespan when feeding on *S. typhimurium.* On the other hand, it also had a short lifespan when feeding on *E. coli*. Therefore, the relative mortality of *ced-9* worms feeding on *S. typhimurium* [defined as (TD_{50} of wild-type worms feeding on *S. typhimurium*/TD_{50} *ced-9* on *S. typhimurium*)/(TD_{50} wild-type worms on *E. coli*/TD_{50} ced-9 on *E. coli*)] was not significantly different from control wild-type worms

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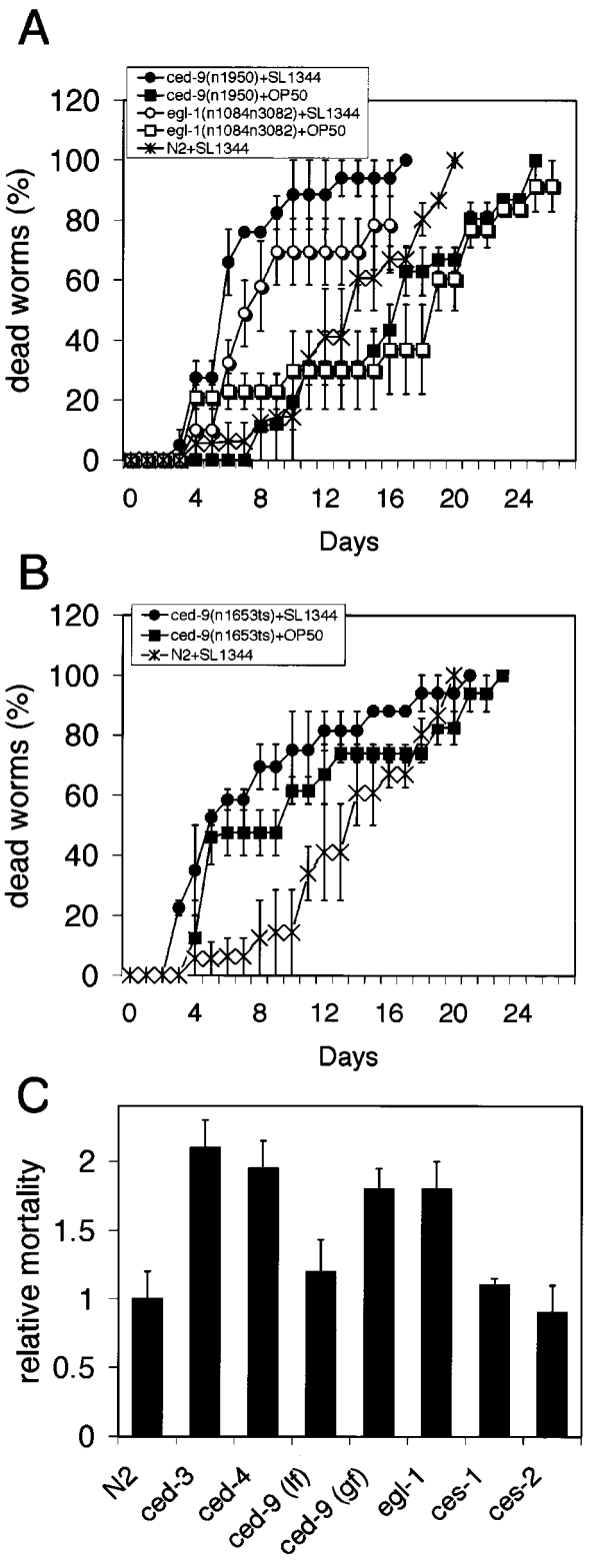

Fig. 3. Mutants deficient in stress-induced germ-line cell death are more susceptible to *S. typhimurium*-mediated killing. One-day-old adult animals were exposed to either *E. coli* or *S. typhimurium*. (*A*) Wild type, *ced-9(n1950) (gf)*, and *egl-1(n1084n3082) (lf)* (*gf,* gain-of-function; *lf,* loss-of-function). *B*, wild type and *ced-9(n1653ts) (lf)*. (*C*) Wild type, *ced-3 (n717)*, *ced-4(n1162)*, *ced-9(n1653ts) (lf)*, *ced-9(n1950) (gf)*, *egl-1(n1084n3082)*, *ces-1(n703)*, and *ces-2(n732)*. The TD_{50}s were calculated in each case and the relative mortality was calculated as described in *Materials and Methods.* More than 10 animals were used in each case; data (mean ± SD) were from three to five experiments.

as illustrated in Fig. 3*C*. The short lifespan of the *ced-9* mutant is most likely related to the fact that loss-of-function mutations in *ced-9* cause sterility and maternal-effect lethality as a consequence of ectopic cell death (14).

Mutations in the genes *ces-1* and *ces-2* affect a specific subset of somatic PCDs (28) but have not been shown to be involved in germ-line cell death (16). As expected, these mutants were not more susceptible than wild-type worms to *Salmonella*-mediated killing (Fig. 3C).

Discussion

In vertebrates, PCD is used to eliminate cells that have been produced in excess, have already served their purpose, or are potentially detrimental to the organism. In *C. elegans*, most cell death seems to belong to the first two categories. However, the data presented in this paper show that *S. typhimurium* colonization of the *C. elegans* intestine leads to an increased level of cell death in the worm gonad and that *C. elegans* PCD mutants are more susceptible to *S. typhimurium*-mediated killing. These results suggest that *S. typhimurium* virulence factors trigger somatic signals that induce the PCD pathway and that induction of the PCD pathway serves a protective role when *C. elegans* encounters an adverse environmental stimulus such as the attack of a potentially pathogenic bacterium. These conclusions are consistent with the following observations. First, when *C. elegans* are colonized by *S. typhimurium*, the intestinal lumen of infected animals is distended and full of intact bacteria, but no bacteria are found beyond this region or in contact with the gonads (1). Second, *C. elegans* PCD-related mutants, including several loss-of-function alleles of *ced-3* and *ced-4* and a gain-of-function allele of *ced-9*, do not exhibit *S. typhimurium*-elicited PCD (Table 1), and are more susceptible to *S. typhimurium*-mediated killing (Figs. 2 and 3). Third, a pleiotropic *S. typhimurium phoP/phoQ* mutant that fails to synthesize a variety of virulence-related factors does not elicit germ-line cell death (Table 1).

Despite the similarities in the regulation of somatic and germ-cell deaths in *C. elegans*, some differences have been found. For example, *ced-9* gain-of-function and *egl-1* loss-of-function mutations, which play key roles in PCD during hermaphrodite development, do not seem to be involved in the type of spontaneous germ-line cell deaths observed when *C. elegans* feeds on *E. coli* (16). However, a recent report demonstrates that they do have a role in stress-induced germ-line deaths. When worms were subjected to γ-irradiation to induce germ-line cell death, the *ced-9 (n1950)* (gain-of-function) mutation abolished radiation-induced germ-cell death, and the *egl-1 (n1084n3082)* mutation reduced it significantly (29). It is possible that both irradiation and *Salmonella* colonization may generate similar noxious stimuli, including oxidative stress, which triggers PCD. It would be interesting to know whether γ-irradiated PCD mutants die more quickly than irradiated wild-type worms. The fact that excess germ cells are eliminated by PCD in starving animals (17) raises the possibility that *S. typhimurium* infection could be causing nutritional stress that leads to germ-line cell death. However, the normal development of worms when feeding on *S. typhimurium* and the rapid induction of cell death by *Salmonella* (Fig. 1*A*) make this possibility unlikely.

In wild-type *C. elegans*, no somatic cell deaths were observed in the soma after the L2 stage and we did not observe any somatic PCD in worms feeding on *S. typhimurium*. However, we cannot rule out the possibility that some somatic cell deaths occur in response to bacterial attack and that these somatic cell deaths play an important role in the defense response to pathogens. It is also possible that *S. typhimurium* colonization activates a *ced-3 ced-4*-dependant signaling pathway that triggers cell death in germ-line cells but activates defense response pathways in somatic cells. Such a pathway, for example, could operate in the *C. elegans* intestine, which is in direct contact with potential bacterial pathogens. At this

Table 1. Relationship between germ cell death and *Salmonella*-mediated killing

Condition	Germ cell corpses*	$n^{\dagger}$	$TD_{50}^{\ddagger}$
N2 + *E. coli* OP50	29 ± 1.8	20	16.8 ± 0.3
N2 + *S. typhimurium* SL1344	91 ± 2.5	20	13.5 ± 1.5
ced-3(n717) + *E. coli* OP50	0.0	20	18.8 ± 0.9
ced-3(n717) + *S. typhimurium* SL1344	0.2 ± 0.4	20	5.8 ± 0.3
ced-4(n1162) + *E. coli* OP50	0.0	15	13.5 ± 1.3
ced-4(n1162) + *S. typhimurium* SL1344	0.0	19	6.9 ± 0.3
ced-9(n1950) + *E. coli* OP50	25 ± 1.4	20	16.1 ± 0.2
ced-9(n1950) + *S. typhimurium* SL1344	29 ± 1.8	20	5.6 ± 0.2
egl-1(n1084n3082) + *E. coli* OP50	28 ± 0.9	20	16.9 ± 1.9
egl-1(n1084n3082) + *S. typhimurium* SL1344	36 ± 1.4	20	7.5 ± 1.1
N2 + *S. typhimurium* SL14028 (wild type)	75 ± 2.3	20	13.0 ± 1.0
N2 + *S. typhimurium* LH954 (*phoP phoQ*)	31 ± 1.7	20	16.5 ± 0.5
N2 + *P. aeruginosa* PA14	30 ± 0.4	15	3.4 ± 0.6

*The germ cell corpses were scored 3 h after the infection of young adult animals by using the vital dye Syto12 as described in *Materials and Methods.* The number of germ corpses per gonad arm was scored only in equally stained animals.

†*n*, number of animals counted.

‡TD_{50}s were calculated as described in *Materials and Methods* and data (mean ± SD) were from duplicates.

point in our investigation, we cannot distinguish between the possibilities that germ-line cell deaths *per se* play a direct protective role or that the *ced-3 ced-4* signal pathway activates unknown defense responses. The mechanism by which germ-line cell death may protect the host from the deleterious effects of pathogen attack is not clear. Nevertheless, one could imagine that there is a selective advantage in regulating the rate of germ-line cell deaths in response to environmental signals.

In contrast to *S. typhimurium*, *P. aeruginosa* does not elicit PCD in *C. elegans* germ cells, and *C. elegans* PCD-related mutants do not exhibit an aberrant phenotype in response to *P. aeruginosa* compared with wild-type worms. A major difference between the *P. aeruginosa* and *S. typhimurium* infection models is that a small inoculum of *S. typhimurium* can proliferate in the intestine of *C. elegans* and establish a persistent and lethal infection even in the presence of a large excess of *E. coli* cells, whereas *P. aeruginosa* accumulates in the intestine only when it is the sole source of food. It is possible that the factors that allow *S. typhimurium* to proliferate in and colonize the intestine may correspond to specific *C. elegans* receptors that in turn may be involved in the elicitation of the PCD response. Consistent with this interpretation, it has been shown recently that PCD induced in lung epithelial cells by *P. aeruginosa* through the CD95/CD95 ligand pathway may protect the host from *P. aeruginosa* infection (30). The absence of *C. elegans* homologues for CD95 could explain why an increase in the PCD rate was not observed in *P. aeruginosa*-infected worms.

In conclusion, our results show that in response to *S. typhimurium* infection, PCD eliminates excess germ cells in the *C. elegans* gonad that could be detrimental to the worms, and that this PCD process may be involved in the *C. elegans* defense response to environmental insults, such as pathogen attack. These findings suggest that PCD is an ancient host defense mechanism that can be readily dissected by using the power of *C. elegans* genetic and genomic analyses.

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