

years 13 through 18. The simulation omitted those years and began with the observed species present when the census was resumed. For Eastern Wood the ninth year was treated similarly. The robin on Skokholm was present only the 12th year (just before the missing censuses) and therefore had no estimated extinction probability. This was arbitrarily reset to .5. The starling on Skokholm was absent before the hiatus but always present afterward. Its estimated immigration probability of 0 was arbitrarily reset to .01. A few species whose presence was questioned in the Skokholm censuses were changed to present, and the dunnoek, which was not censused the first year to Eastern Wood but was present, usually with several breeding pairs

- during 23 of the 25 remaining years, was assumed to be present initially. Simulations were run with all of these conventions modified in a variety of biologically reasonable ways, with no substantive change in the results.
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## Mutations Affecting Programmed Cell Deaths in the Nematode *Caenorhabditis elegans*

**Abstract.** *Mutations in two nonessential genes specifically block the phagocytosis of cells programmed to die during development. With few exceptions, these cells still die, suggesting that, in nematodes, engulfment is not necessary for most programmed deaths. Instead, these deaths appear to occur by cell suicide.*

During development, the nematode *Caenorhabditis elegans* follows an essentially invariant pattern of cell divisions which produces cells of rigidly specified fates (1-3). One fate is for cells to die, usually within 1 to 2 hours of their birth, in mitosis. For example, of 671 cells produced during embryogenesis of the hermaphrodite, 113 cells die before hatching. An additional 18 cells die during larval growth as the number of somatic nuclei increases from 558 to an eventual 959. Normally, these cells are engulfed and degraded by neighboring cells at the time of their deaths (1, 4). Mutations in two genes, described below, prevent the elimination of dead cells by blocking their phagocytosis. With certain exceptions, cells die at their normal times in these mutants, suggesting that most programmed deaths occur by cell suicide, not from phagocytosis. Neither mutation disrupts development.

Cell divisions and deaths can be observed in living nematodes by Nomarski differential interference contrast microscopy (2). Overt signs of cell death, a darkening of the cytoplasm and nucleoplasm in electron micrographs or a corresponding increase in refractility in Nomarski optics, appear 30 minutes or more after cytokinesis (1-4). A period of high, uniform refractility, during which the entire cell has the appearance of a flat raised disk in Nomarski optical section, persists for 10 to 30 minutes. Engulfed cells then lose refractility and shrink, eventually disappearing. The entire process, from the first increase in refractility to the disappearance of the cell, takes about an hour. A sequence of Nomarski photographs showing the death of a presumptive ventral cord motor neuron is given in Sulston and Hor-

vitz (2). A corresponding series of electron micrographs is shown in (4).

Mutations were induced in hermaphrodites by exposure to ethyl methanesulfonate (5). The F<sub>2</sub> progeny were screened under Nomarski optics for abnormal persistence of embryonic cell deaths. Eight independent strains were obtained in which dead cells were not

resorbed. We designated these *ced* mutants, mnemonic for programmed cell death. All eight mutations were recessive and, together, defined two complementation groups, *ced-1* (*el735*, *el754*, *el797*, *el798*, *el799*, *el801*, and *el814*) and *ced-2* (*el752*).

Mutations *el735* (*ced-1*) and *el752* (*ced-2*) were mapped to linkage groups I and IV, respectively. Recombination frequencies were determined from cis double heterozygotes. The distances, given in recombinant chromosomes per total chromosomes examined, were *dpy-5* (*e61*) *ced-1*, 2/24; *unc-75* (*e950*) *ced-1*, 0/24; and *ced-2* *dpy-13* (*el84*), 5/24. Trans three-factor crosses gave the following gene orders: *dpy-5*[*unc-13*(*e51*),*ced-1*], 12 recombinants; *unc-75*(1/21)*ced-1*(20/21) *lev-11*(*x12*); [*ced-2*,*unc-17*(*el13*)]*dpy-13*, 5 recombinants; and *dpy-9*(*el2*)(13/23) *ced-2*(10/23)*lin-1*(*el275*).

The phenotypes of *ced-1* (*el735*), *ced-2* (*el752*), and the *ced-1*, *ced-2* double mutant are indistinguishable by Nomarski and electron microscopy and by Feulgen staining, suggesting that *ced-1* and *ced-2* mutations affect closely related steps in the removal of dead cells, though neither gene product can substitute for the other.

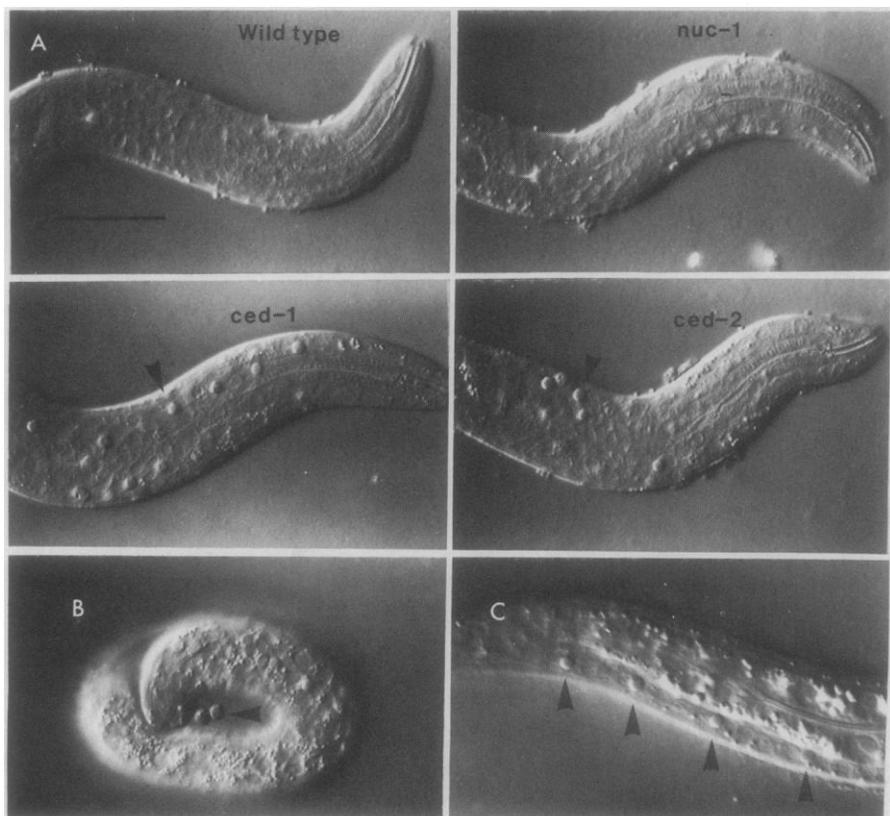


Fig. 1. (A) Nomarski photographs of newly hatched wild-type (N2), *nuc-1* (*el392*), *ced-1* (*el735*), and *ced-2* (*el752*) larvae showing normal cell nuclei and refractile cell deaths (arrows) in the head. (B) Embryonated egg (*el735*) with two dead cells shed into the egg fluid (arrow). (C) Posterior ventral nerve cord of 15-hour first-stage larva (*el752*). Arrows mark (from right to left) refractile dying cells P9.aap, P10.aap, P11.aap, and P12.aap [for nomenclature, see (2)]. Scale bar, 20  $\mu$ m.

Most embryonic cell deaths occur between 5 and 9 hours after fertilization. By 12 hours, the time of hatching, no trace of these cells remains in wild-type animals (1). By contrast, in late-stage embryos (pretzels) and newly hatched larvae of *ced-1* and *ced-2* mutants, many of these cells persist, arrested at the highly refractile stage in the normal progression of programmed death (Fig. 1A). We have determined, by electron microscopy of newly hatched animals, that these cells are not engulfed (Fig. 2). Frequently, a small number of the most superficial dead embryonic cells are actually shed into the egg fluid surrounding the embryo (Fig. 1B). Postembryonic

cell deaths are also persistent (Fig. 1C) and, of these, all deaths in both sexes are affected.

We examined particular embryonic and postembryonic cell deaths in greater detail in the strains *ced-1* (*el735*) and *ced-2* (*el752*). The first signs of impending death—increases in cytoplasmic and nuclear refractility—occurred at normal times in both strains. Once obtained, however, the highly refractile stage usually persisted for several hours and, occasionally, throughout larval development. Dead cells eventually shrank away but the precise timing varied greatly, even for the same cell. In some instances [for example, the deaths of embryonic

cells AB.arpaaapp and AB.plpappap described in (1)], it was possible to observe the details of cell engulfment in living animals by Nomarski microscopy. Whereas in wild-type embryos dying cells were quickly engulfed and degraded, in mutant embryos engulfing was much less reliable. If engulfed, the cells were rapidly degraded, but many dead cells escaped engulfment in these mutants and could persist indefinitely. Sometimes such cells drifted about in the embryo and were eventually engulfed by a more distant cell than their usual neighbor. Hence, the wild-type *ced-1* and *ced-2* products were not essential for phagocytosis but appeared to enhance the efficiency of engulfment. We do not know whether these products are required in the dying cells or their engulfing partners; this question can perhaps be decided in the future by creating genetic mosaics.

Because engulfment is concomitant with normal deaths (4), it has been speculated that engulfment is a prerequisite for death (4, 6). This is probably incorrect for most deaths. In favorable cases, embryonic cells could actually be seen to die before being engulfed. Similarly, most deaths occurred with normal timing in *ced-1* and *ced-2* mutants although engulfing was abnormal. Two cells in the *C. elegans* male are known, however, which will not die unless engulfed by specific neighbors (6). These cells will survive indefinitely if their designated killer cells are selectively removed by laser microablation. In *ced-1* and *ced-2* strains, such cells probably survive when engulfing fails. In particular, the cells B.a<sup>1</sup>/<sub>r</sub>apaav have both been observed to survive in individual mutant males, presumably because their designated killer cell, P12.pa, failed to engulf. [For nomenclature, see (6).] Thus, although most programmed deaths in *C. elegans* probably occur through suicide, certain deaths require engulfment by specific killer cells. The wild-type *ced-1* and *ced-2* products are required, in either case, for the process of engulfment, not killing.

Sulston (7) has described a mutant [*el392* (*nuc-1*)] on linkage group X that lacks endodeoxyribonuclease. In these animals, the DNA from dead cells is not degraded but persists as a compact dot of Feulgen-reactive material. By Nomarski criteria, however, cell deaths are normal in these animals and the bulk of the nucleoplasm is effectively removed (Fig. 1A). In *ced-1* and *ced-2* mutants, by contrast, neither DNA nor nucleoplasm is removed (Figs. 1 and 3). Moreover,

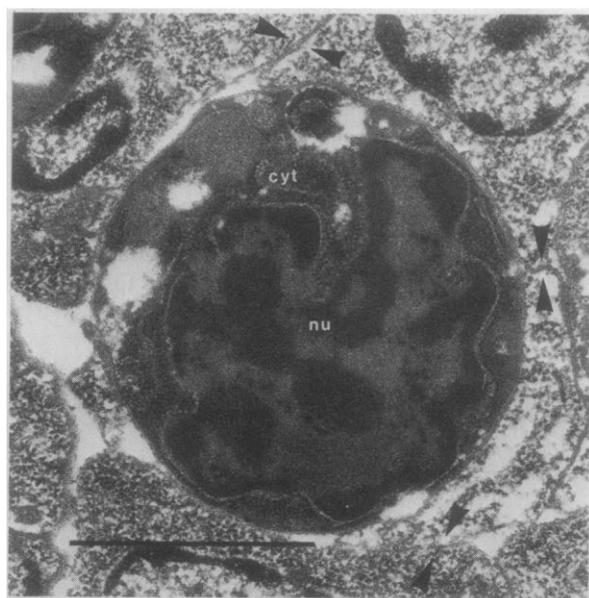


Fig. 2. Electron micrograph of a persistent, unengulfed dead cell in a newly hatched *ced-2* (*el752*) larva. Four healthy neurons are pressed against the dead cell. Paired arrows show the appositions of their cell membranes. Letters mark the nucleus (*nu*) and cytoplasm (*cyt*) of the dead cell. The animal was cut and fixed in 2.5 percent glutaraldehyde in 0.1M Hepes buffer, pH 7.5, for 1 hour, washed, and then post-fixed with 1 percent buffered osmium tetroxide. Scale bar, 1  $\mu$ m.

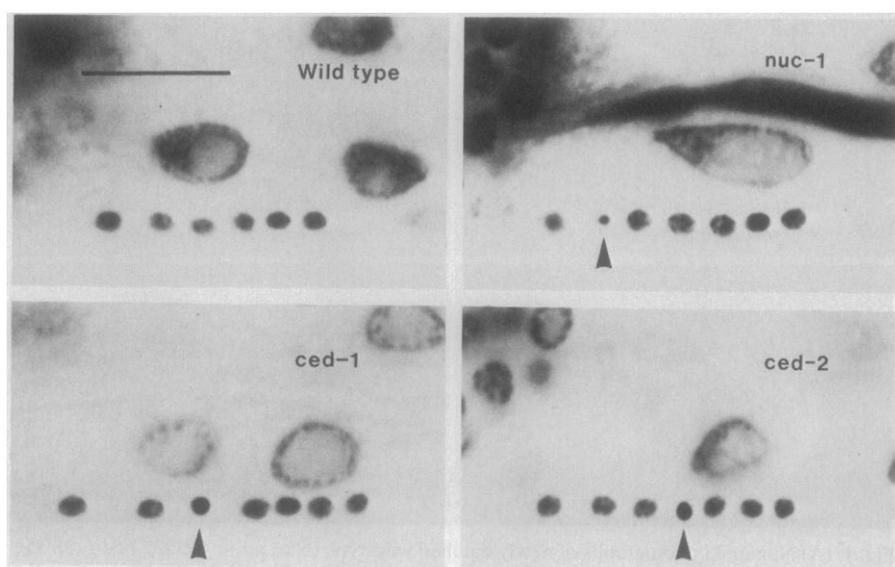


Fig. 3. Feulgen-stained ventral nerve cords showing abnormally persistent DNA from dead P10.aap cells (arrows). The very large nuclei above the neurons are in intestinal cells. The dark horizontal stripe in the *nuc-1* animal is due to undegraded bacterial DNA in the intestinal lumen. Left lateral views of fourth-stage hermaphrodites. Scale bar, 10  $\mu$ m.

the DNA in dead cells in these mutants is actually less condensed and more persistent than in the *nuc-1* strain. Since the *ced-1* and *ced-2* strains have normal endodeoxyribonuclease activity (8), we conclude that the enzyme does not gain access to the DNA in these cells. In a formal sense, the *ced-1* and *ced-2* products act before the *nuc-1* enzyme. In particular, it suggests that the endonuclease is normally provided by the lysosomes of the engulfing cell and not by the dying cell. It leaves open the intriguing question of what biochemical machinery is used by dying cells to reach the refractile stage characteristic of unengulfed deaths.

Chalfie and Sulston (9) have described a dominant mutant [*el611* (*mec-4*)] in which a class of six homologous mechanosensory neurons die after initial differentiation. These deaths differ from normal programmed cell deaths in that the cells never become refractile and, very frequently, a large nonrefractile vacuole forms around the dead cell. We examined the degeneration of these neurons in double mutants homozygous for *el611* and either *ced-1* or *ced-2* and found no differences from *el611* mutants alone. This suggests that the wild-type *ced-1* and *ced-2* products are not involved in clearing these abnormal deaths. Similarly, these products do not speed the removal of cells killed unnaturally by the laser microbeam technique (10).

Most deaths occur in neuronal lineages and many of the dying cells would be expected to be neurons. Death may occur before (4) or after extension of neurites (1). In certain cases, we have a good guess for the potential fates of these cells. For example, six sex-specific neurons (four male-specific neurons and two hermaphrodite-specific neurons) are produced in embryos of either sex but only neurons appropriate to the sex of the embryo are retained; the others undergo selective death (1). Here we surmise that the cells are fated to be neurons of a particular class with sex-specific death as an overriding fate.

Ellis and co-workers (11) recently discovered an additional gene involved in cell death by selecting an apparent revertant of *ced-1*. Mutations in *ced-3* block the initiation of cell deaths and allow doomed cells to differentiate and assume their presumptive underlying fates.

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## The Genus-Specific Antigen of *Chlamydia*: Resemblance to the Lipopolysaccharide of Enteric Bacteria

**Abstract.** A strong immunological cross-reaction between a major glycolipid antigen of *Chlamydia* and the innermost (*Re*) core of the lipopolysaccharide of enteric bacteria was demonstrated with the aid of mutants in which the *Re* structure is exposed. The chlamydial glycolipid resembled the *Re* lipopolysaccharide in molecular size, solubility, and endotoxic properties and may thus be functionally equivalent to lipopolysaccharide, an essential and characteristic component of the outer membrane of Gram-negative bacteria.

Chlamydiae are important pathogens of man and many animals, yet their biological nature has been elusive and indeed they have only recently been recognized as bacteria. They have an unusual developmental cycle (1) with an intracellular growth form and an extracellular infective form. In both forms the chlamydial cells are surrounded by two membrane layers (2), an arrangement typical of Gram-negative bacteria; however, the importance of this morphological feature is difficult to evaluate in organisms lacking the most distinctive layer of the bacterial cell envelope, the peptidoglycan. The presence of lipopolysaccharide (LPS), a typical constituent of the outer membrane of Gram-negative bacteria, has been suggested in chlamydiae on the basis of the pyrogenic properties of chlamydiae and Gram-negative bacteria and because of the immunological relatedness of their lipid components

(3, 4). The sugar, 3-deoxy-manno-octulosonic acid (KDO, or ketodeoxyoctulosonic acid), is a constituent of the LPS in most Gram-negative bacteria (5). The same sugar has been found in the "genus-specific" glycolipid antigen of chlamydiae, present in both known chlamydial species (*Chlamydia trachomatis* and *C. psittaci*) and throughout the growth cycle (6). We report that this glycolipid resembles in several parameters and most notably in its immunological properties the inner core of enterobacterial LPS. These data strongly support the view of the chlamydial outer membrane as equivalent to the outer membrane of usual Gram-negative bacteria.

The structure of LPS has been most thoroughly studied in the enteric bacteria *Salmonella* and *Escherichia coli* (5). The LPS molecule consists of three different domains (Fig. 1). The lipid A part is responsible for the endotoxic activity of

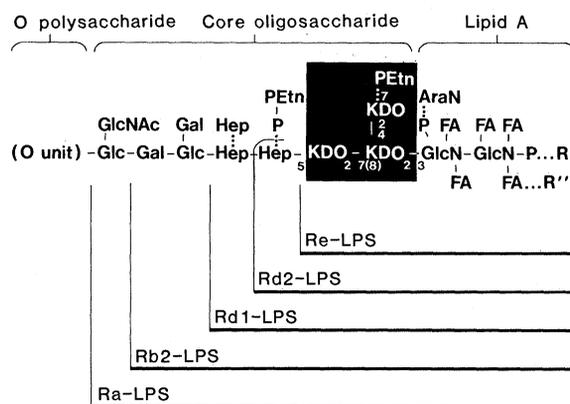


Fig. 1. Schematic structure of *Salmonella* LPS (5, 16). The linkages in the KDO region are partly inferred from studies with *E. coli* (16). The KDO-trisaccharide is shaded. The LPS of wild-type bacteria has the complete structure, whereas rough mutants have various abbreviated forms (Ra to Re), depending on the biosynthetic block in each case (8). Less than stoichiometric substitutions are shown by dotted lines. Abbreviations: Ac, acetyl; Etn, ethanolamine; FA, fatty acid; KDO, 3-deoxy-D-manno-octulosonic acid; P, phosphate; R' and R'', variable substituents.