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18. Binding to intact cells was carried out in modified KRB (*12*) for 20 min at room temperature and binding to membranes in 25 mM Hepes, 1% albumin, 5 mM $MgCl_2$, pH 7.7, for 1 hour at 4°C. Specific binding was defined by the displacement with diprenorphine.
19. RNA was isolated using the Fast Track mRNA Isolation Kit (Invitrogen), and Northern blot analysis performed at 65°C as described [T. G. Boulton *et al.*, *Cell* **65**, 663 (1991)], followed by autoradiography for 1 week at −70°C with an enhancing screen. Repeated studies consistently showed a wide range of transcripts, with no evidence for RNA degradation either by staining the gels with ethidium bromide or by rehybridization with a probe for β actin. Southern blots were washed in 0.2× standard sodium citrate, 0.1% SDS at 55°C.
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# Prevention of Programmed Cell Death in *Caenorhabditis elegans* by Human *bcl-2*

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**Programmed cell death is a physiological process that eliminates unwanted cells. The *bcl-2* gene regulates programmed cell death in mammalian cells, but the way it functions is not known. Expression of the human *bcl-2* gene in the nematode *Caenorhabditis elegans* reduced the number of programmed cell deaths, suggesting that the mechanism of programmed cell death controlled by *bcl-2* in humans is the same as that in nematodes.**

Cell death is a normal part of development and homeostasis in both vertebrates and invertebrates (*1*). Extensive loss of neurons occurs during vertebrate development (*2*), and in mature animals cell death serves to eliminate autoreactive immune cells and virally infected cells (*3*). In mammals, *bcl-2* prevents some, but not all, cell deaths (*4–7*). For instance, expression of the *bcl-2* gene can prevent the death of myeloid or neuronal cells that occurs after withdrawal of certain growth factors (*4*), but it cannot protect cells from being killed by cytotoxic T cells (*7*). Inhibition of cell death by improperly regulated expression of *bcl-2* may also be oncogenic, as when *bcl-2* is activated by t(14;18) translocations in human follicular lymphoma (*8*) or when *bcl-2* is overexpressed in transgenic mice (*5*). The activation and execution of an intracellular suicide pathway is referred to as programmed cell death (*1*).

Programmed cell death occurs in the nematode *Caenorhabdits elegans*. Of the 1090 somatic cells formed in the course of the development of an adult worm, 131 cells undergo programmed cell death (*9*), and the genetic pathway by which cell death occurs has been extensively characterized (*1*). We tested whether programmed cell deaths in nematodes and humans can occur by way of the same molecular pathway by constructing a vector (*hsbcl-2*) that places expression of the human *bcl-2* gene under control of the nematode heat shock promoter (*10*) and generating transgenic nematodes with *rol-6* as a cotransformation marker (*11, 12*). Normally, cells undergoing programmed cell death are observable only for a short period, because the cell corpse is rapidly engulfed (*9*). We therefore used *ced-1(e1735)* mutants because this mutation prevents engulfment, allowing the cell corpses to accumulate (*13*).

We used antibody staining to demonstrate that transgenic nematode embryos express the human *bcl-2* protein. After heat shock, embryos from transgenic parents were fixed and labeled with monoclonal antibodies to human *bcl-2* (*14*), and then staining was visualized by fluorescence microscopy (*15*). Transgenic sequences in nematodes are carried and transmitted as an extrachromosomal array (*11*), so we expected to see *bcl-2* expression in those progeny that carried the transgene, and not in their nontransgenic siblings. A proportion of offspring from transgenic parents were positive for *bcl-2* staining (Fig. 1). Staining was most prominent in the perinuclear regions of the cytoplasm, remained strong for up to 8 hours after heat shock, and was still

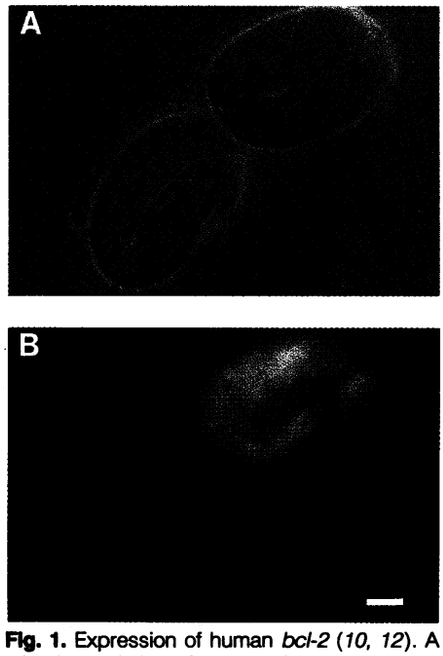


**Fig. 1.** Expression of human *bcl-2* (*10, 12*). A mixed population of transgenic and nontransgenic sibling embryos from *hsbcl-2* parents were stained with antibodies to human *bcl-2* and examined by phase contrast (**A**) and fluorescence microscopy (**B**) (*15*). Expression of *bcl-2* is seen in one of two embryos. Scale bar, 10 μm.

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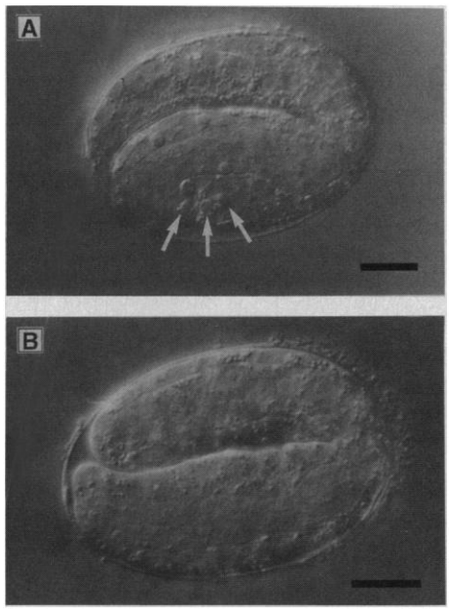


**Fig. 2.** Corpses of cells that have undergone programmed cell death are visible as highly refractile nuclei (*16*). (**A**) An early threefold stage embryo that is a nontransgenic offspring of an *hsbcl-2* parent. Three of the seven cell corpses visible in this focal plane are indicated by arrows. (**B**) An *hsbcl-2* embryo. No corpses are evident in a comparable focal plane. Scale bar, 10 μm.

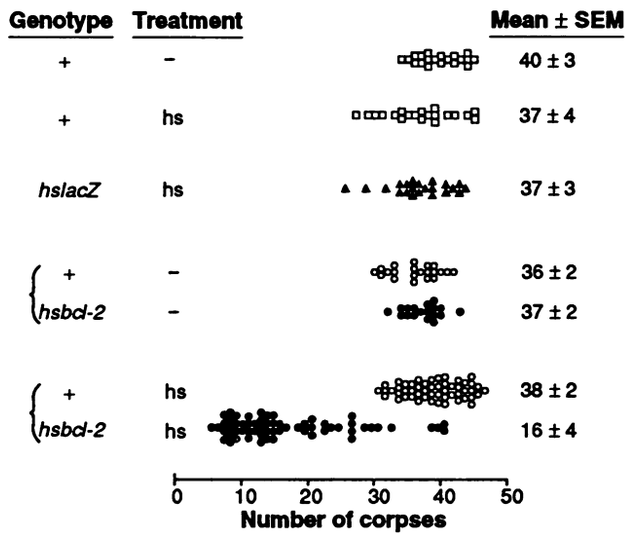


**Fig. 3.** Expression of *bcl-2* prevents programmed cell deaths. The total number of corpses seen in embryos at 520 min of development is shown. Each symbol represents a single embryo. *ced-1* embryos (open squares); *ced-1*; *hslacZ* embryos (filled triangles); *ced-1* embryos that are the siblings of *ced-1*; *hsbcl-2* embryos (open circles); *ced-1*; *hsbcl-2* embryos (filled circles). Nontransgenic animals (+); transgenic animals carrying the *rol-6* and the *hslacZ* transgenes (*hslacZ*); transgenic animals carrying the *rol-6* and *hsbcl-2* transgenes (*hsbcl-2*) (*17*). Embryos that had been subjected to a heat shock treatment (hs) (*16*); embryos that were grown at 20°C continuously (−). The brackets indicate progeny that were derived from the same parents and were given identical treatment. Similar results were obtained using an independent *hsbcl-2* strain.

detectable 12 hours after heat shock. This staining was due to expression from the *hsbcl-2* gene, because staining was not detected in nontransgenic embryos nor in transgenic animals that had not been subjected to heat shock.

We observed that *bcl-2* expression significantly reduced the number of cell corpses. Embryos were subjected to heat shock at the early gastrulation stage, before the onset of any cell deaths. Development was then allowed to proceed until morphogenesis was complete, by which time almost all of the embryonic cell deaths would normally have occurred (*16*). We then examined individual animals for the number of cell corpses (Fig. 2). Subsequently, we determined which of the animals contained transgenic sequences, on the basis of expression of the roller phenotype (*17*). Nontransgenic progeny exhibited the same number of cell corpses [38 ± 2 corpses (mean ± 3 SEM)] as were observed in the *ced-1* strain (37 ± 4 corpses). However, embryos that carried *hsbcl-2* and *rol-6* contained a significantly lower number of corpses (16 ± 4 corpses; $P < 0.0001$) (Fig. 3). Control experiments showed that this reduction in cell corpses was due to expression of *bcl-2*, because the reduction in the number of cell corpses was not a result of expression from the *rol-6* marker or heat shock.

Continuous observation of several *hsbcl-2* animals throughout embryogenesis showed that the cell death rate was low, but each corpse persisted for a relatively long time. This means that the reduction in the number of corpses in *hsbcl-2* animals was due to a lower number of cell deaths and not rapid engulfment of cell corpses. Furthermore, we saw no increase in the numbers of corpses at later times, suggesting that expression of the human *bcl-2* gene prevents cell deaths rather than merely delaying the onset of cell death.

The conservation of function of *bcl-2* suggests that the mechanisms that execute programmed cell death are basic and are used in diverse organisms. In order to prevent cell deaths in *C. elegans*, the human *bcl-2* protein most likely interacts with the gene products that mediate programmed cell death in *C. elegans*. Of the cell death genes already identified, the genetic behavior of *ced-9* is the most similar to that of *bcl-2*. Gain-of-function *ced-9* mutations in *C. elegans* (*18*) and expression of *bcl-2* in mice (*5*) both prevent programmed cell deaths. As *ced-9* appears to act by inhibiting *ced-3* or *ced-4* activity (*19*), *bcl-2* most likely works in humans by antagonizing the activity of their homologs.

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15. Unstaged embryos from *hsbcl-2* parents were subjected to a heat shock (15 min at 33°C) and allowed to express the *hsbcl-2* transgene (2 hours at 20°C). The embryos were fixed in methanol and acetone and then stained for 1 hour with a monoclonal antibody to human *bcl-2* (Dako, Glostrup, Denmark) (*14*) diluted 1:40 in phosphate-buffered saline with 5% albumin. This was followed by staining for 30 min with a 1:100 dilution of fluoresceinated goat anti-mouse antibodies (Caltag, South San Francisco,

CA). Perinuclear staining was confirmed by counterstaining with propidium iodide.
16. Because most cell deaths (109 out of 131) occur 4 to 8 hours after fertilization (*9*), a heat shock was given to embryos at about 1 hour of development. Early embryos from *hsbcl-2* parents were collected from adults that had been cut open with a needle. The embryos were allowed to develop until early gastrulation (30 min at 20°C) and subjected to a heat shock (15 min for 33°C). The embryos were allowed to develop until the end of morphogenesis (7 to 9 hours at 20°C). Individual embryos were placed on an agar pad and photographed using Nomarski optics microscopy (*9*).
17. Only some embryos from *hslacZ* and *hsbcl-2* parents carry transgenic sequences, corresponding to the fraction of progeny inheriting an extrachromosomal array. In these cases, the *rol-6* marker gene was used to determine the genotype of each embryo; embryos that grew into adults with a roller phenotype or that segregated progeny with a roller phenotype were scored as transgenic. Some animals died due to the heat shock treatment, and the data from these animals were not included in this experiment.
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# Neutrophil Recruitment by Tumor Necrosis Factor from Mast Cells in Immune Complex Peritonitis

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**During generalized immune complex–induced inflammation of the peritoneal cavity, two peaks of tumor necrosis factor (TNF) were observed in the peritoneal exudate of normal mice. In mast cell–deficient mice, the first peak was undetected, and the second peak of TNF and neutrophil influx were significantly reduced. Antibody to TNF significantly inhibited neutrophil infiltration in normal but not in mast cell–deficient mice. Mast cell repletion of the latter normalized TNF, neutrophil mobilization, and the effect of the antibody to TNF. Thus, in vivo, mast cells produce the TNF that augments neutrophil emigration.**

Leukocyte-endothelial cell interaction is a pivotal event in leukocyte emigration from the intravascular to the interstitial space during inflammation. TNF-α enhances neutrophil adhesion to endothelial cells in vitro. This is due, in part, to the expression of the adhesion proteins E-selectin (ELAM-1) and ICAM-1 by endothelial cells. The same cytokine also stimulates various cell types to release the neutrophil chemotactant interleukin-8 (IL-8). TNF-α also seems to be directly chemotactic to certain leukocytes, producing the up-regulation of integrin expression on the cell surface of leukocytes as well as the activation of these cells (*1*). Macrophages, neutrophils, T lymphocytes, natural killer (NK) cells, and mast cells can be stimulated to synthesize TNF-α (*1*). However, only mast cells store TNF-α in their granules. Activated mast cells, therefore, release TNF-α immediately from preformed stores and later release newly synthesized TNF (*2*). This suggests that TNF-α derived from mast cells may participate in the early phase of inflammation. Mast cell degranulation, TNF-α, and venular endothelial cell expression of E-selectin are correlated in human skin organ cultures exposed to morphine (*3*). Mast cells can also generate various other cytokines, including IL-1 (*4*). These cells are strategically located in high density around blood vessels, especially postcapillary venules, which are the principal sites of plasma flux and leukocyte transmigration into the interstitium. Mast cells are involved in neutrophil elicitation, plasma exudation, edema and fibrin deposition in reverse Arthus reaction [which is characterized by generalized antigen-antibody complex formation (*5–7*)] and antibody-mediated basement membrane injury (*8, 9*) in the skin, and neutrophil influx in thioglycollate- and immune complex–induced peritonitis (*10, 11*). Mast cells are also involved in inflammation induced by phorbol myristate acetate and substance P (*12, 13*) and in antigen-induced arthritis (*14*). The present study shows the significance of mast cell–generated TNF-α in the recruitment of neutrophils in immune complex–mediated inflammation.

Reverse passive Arthus reaction in the peritoneal cavity (intravenous injection of antigen and intraperitoneal injection of antibody) of mast cell–deficient $WBB6F_1$-$W/W^v$ ($W/W^v$) mice and their congenic controls, $WBB6F_1$-+/+ (+/+), was chosen as the model of inflammation for the evaluation of the release and role of TNF-α, with mast cells as the possible source of TNF-α. Neutrophil accumulation was time-dependent and reached a maximum at 6 hours in both sets of mice (Fig. 1). However, the leukocyte influx was reduced in $W/W^v$ mice, and in +/+ mice significant neutrophil infiltration was observed earlier (15 min) than in the $W/W^v$ mice (30 min). Mast cells seemed to account for at least 40% of the neutrophil response. Correction of the mast cell deficiency of $W/W^v$ mice

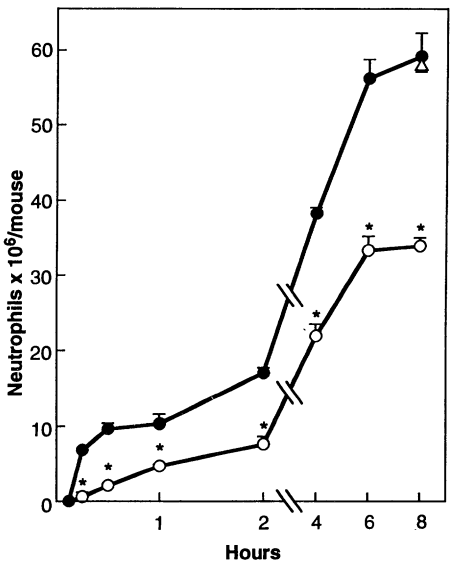


**Fig. 1.** Neutrophil influx in peritoneal reverse passive Arthus reaction in normal and mast cell–deficient mice. $WBB6F_1$-$W/W^v$ (○), normal congenic control $WBB6F_1$-+/+ (●) (*21*), and mast cell–reconstituted $W/W^v$ (△) (*11*) mice were anesthetized with pentobarbital (40 to 50 mg per kilogram of body weight, intraperitoneally) and intravenously injected with chicken ovalbumin (20 mg/kg). The mice were then immediately injected (intraperitoneally) with rabbit immunoglobulin G (IgG) to chicken ovalbumin (800 μg per mouse) or phosphate-buffered saline (PBS). At the times indicated after challenge, mice were asphyxiated with $CO_2$, and their peritoneal cavity lavaged with 1 ml of PBS containing 0.1% bovine serum albumin. The protocol used in the care of the mice was in accordance with institutional guidelines. The peritoneal fluid was centrifuged at 200*g* for 10 min. Neutrophil influx was evaluated by measurement of myeloperoxidase (MPO) activity colorimetrically in the cell lysate (*22*). The number of neutrophils present was determined from a standard curve of MPO activity of known numbers of neutrophils (obtained from peritoneal exudate after 6 hours of reverse Arthus reaction). MPO activity in peritoneal cells of PBS-treated mice was equivalent to $0.7 \times 10^6$ neutrophils. Data are expressed as mean ± SEM ($n = 3$ to 6); *$P < 0.01$, comparison of +/+ and $W/W^v$ mice at the respective time points, as determined by the Student's $t$ test.

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