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Do all programmed cell deaths occur via apoptosis?

(T cell/*Manduca*/intersegmental muscle/ubiquitin)

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ABSTRACT **During development, large numbers of cells die by a nonpathological process referred to as programmed cell death. In many tissues, dying cells display similar changes in morphology and chromosomal DNA organization, which has been termed apoptosis. Apoptosis is such a widely documented phenomenon that many authors have assumed all programmed cell deaths occur by this process. Two well-characterized model systems for programmed cell death are (*i*) the death of T cells during negative selection in the mouse thymus and (*ii*) the loss of intersegmental muscles of the moth *Manduca sexta* at the end of metamorphosis. In this report we compare the patterns of cell death displayed by T cells and the intersegmental muscles and find that they differ in terms of cell-surface morphology, nuclear ultrastructure, DNA fragmentation, and polyubiquitin gene expression. Unlike the T cells, which are known to die via apoptosis, we find that the intersegmental muscles display few of the features that characterize apoptosis. These data suggest that more than one cell death mechanism is used during development.**

Any cell can be killed by a wide range of pathological stimuli, such as certain toxins or viruses. These murdered cells die by a process known as necrosis, which involves disruption of membrane integrity and subsequent cellular swelling that results in lysis. In contrast, programmed cell death (PCD) involves the stereotypic loss of individual cells at specific times during development (1). Unlike necrosis, which is a passive process, PCD requires *de novo* gene expression (for review, see refs. 1 and 2).

PCD serves many required functions during normal animal development (3–5). In many tissues, more cells are produced than are ultimately required by the organism, and the excess cells subsequently die by means of PCD. In some cases, the overproduction of cells provides the organism with valuable developmental plasticity. For example, more spinal motor neurons are produced in the chicken embryo than are required to innervate potential peripheral targets (6). This excess ensures that adequate innervation is always available, independent of the size of the muscle encountered. In other situations, cells are removed because they present a threat to the developing organism. The best-characterized example of this phenomenon is the death of immature T cells in the mouse thymus (7, 8). The T-cell receptor on many immature T cells can recognize self-antigens present within the mouse. Should these cells be allowed to survive and proliferate, an autoimmune disorder could ensue. The immune system avoids this potentially lethal situation by removing self-reactive T cells by a process known as negative selection (9–11). Experimentally, the synchronized death of entire populations of immature T cells can be induced with glucocorticoids (12). Much of the data obtained during the study of apoptosis has been acquired with T cells treated with the synthetic glucocorticoid dexamethasone.

A second developmental strategy is to produce cells that differentiate and assume some required, but transient, function and are then removed when the life style of the organism changes. One well-characterized example is the death of the intersegmental muscles (ISMs) during metamorphosis in moths (13, 14). These embryonically derived muscles are used for various locomotory and defensive behaviors in the larva and pupa and in the eclosion (emergence) behavior of the adult. The ISMs are not required for adult-specific behaviors and die during the 30 hr after eclosion. The trigger for this PCD is a fall in the steroid molting hormone 20-hydroxyecdysone late on the day preceding eclosion (15).

Kerr *et al.* (16) observed that the ultrastructure of most mammalian cells undergoing PCD change similarly, a process they termed apoptosis. They then suggested that perhaps all PCD occurred via apoptosis (17). This supposition has become so widely accepted that many authors use these terms interchangeably. During an examination of the ISMs, we realized that although these cells clearly undergo PCD, they displayed few of the features currently held to be characteristic of apoptosis, such as membrane blebbing, chromatin condensation, and DNA fragmentation (18). Additionally, although the death of both T cells and the ISMs require *de novo* gene expression (19, 20), only the ISMs exhibit dramatic increases in expression of the polyubiquitin gene. Taken together, these data suggest that some cells may use a PCD mechanism that is distinct from apoptosis.

EXPERIMENTAL PROCEDURES

Animals. The tobacco hawkmoth, *Manduca sexta*, was reared and staged as described (15). In some experiments, day 17 developing adults were injected with 10 μl of 20-hydroxyecdysone at 2.5 μg/μl to delay the time of ISM degeneration.

BALB/c mice were reared under standard laboratory conditions. In some experiments, animals received an i.p. injection of 250 μg of dexamethasone in 2.0 ml of phosphate-buffered saline. The thymus was removed from two individual 6- to 8-week-old males, and the thymocytes were released by gently grinding the tissue between two glass microscope slides.

The mouse T-cell hybridoma cell line D011.10 (21) was grown *in vitro* in Dulbecco's modified Eagle's medium (DMEM) (5 g/liter)/RPMI 1640 medium (GIBCO; 5 g/liter)/glucose at 3 g/liter/sodium bicarbonate at 2.85 g/liter, pH 6.9/10% horse serum/gentamicin sulfate at 20 μg/ml/2 mM glutamine.

Electron Microscopy. Cells were fixed in 2% gluteraldehyde/2% formaldehyde in 0.1 M cacodylate buffer/0.1 M NaCl/0.2% $CaCl_2$ and then postfixed in 2% OsO_4. Cells were

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Abbreviations: ISM, intersegmental muscle; PCD, programmed cell death.

then either critically point dried for scanning EM or embedded for transmission EM.

DNA Isolation and Analysis. At various stages of development, cells were removed from the animal, and chromosomal DNA was isolated. Cells were washed in saline and gently homogenized in extraction buffer (proteinase K at 0.1 mg/ml/100 mM NaCl/10 mM Tris·HCl, pH 8.0/25 mM EDTA, pH 8.0/0.5% SDS). The samples were incubated overnight at 50°C. After digestion, the samples were phenol/chloroform extracted, ethanol-precipitated, and resuspended in 10 mM Tris·HCl, pH 8.0/10 mM EDTA, pH 8.0 (TE). DNA (2.5 μg per stage for thymocytes; 5 μg per stage for the ISMs) was fractionated in 2% agarose and stained with ethidium bromide (10 μg/ml).

The agarose gel of *Manduca* DNA was transferred by capillarity in 0.4 M NaOH to Zeta-Probe nylon membrane (Bio-Rad). The filter was baked for 2 hr at 80°C and was hybridized overnight at 65°C with ^{32}P-labeled *Eco*RI-digested *Manduca* genomic DNA under stringent conditions according to the manufacturer's specifications.

RNA Isolation and Analysis. Cells were homogenized with a "tissuemizer" (Tekmar, Cincinnati) in 4 M guanidinium isothiocyanate, and total RNA was isolated by centrifugation through 5.7 M CsCl, as described in ref. 20. For each stage, 15 μg of total RNA was denatured in formaldehyde and separated in 1.5% agarose before transfer to Zeta-Probe membrane. RNA was hybridized with a ^{32}P-labeled cDNA clone 3JSF, which encodes the mouse polyubiquitin gene (22).

RESULTS

Changes in Surface Morphology Accompanying Cell Death. Using scanning EM, we examined the surface morphology of normal and dying cells (Fig. 1). Control D011.10 T-cell hybridoma cells are approximately spherical and possess many surface villi (Fig. 1*A*). These cells can be induced to die via apoptosis in response to a wide variety of stimuli—including dexamethasone, anti-CD3 antibody, or the anti-T cell receptor antibody F23.1 (ref. 21; S.W.S. and B.A.O., unpublished work). When D011.10 cells die in response to dexamethasone, they shrink, and the membrane displays a "boiling" morphology (Fig. 1*B*). Large numbers of protuberances form that appear to pinch off from the surface of the cell. These surface protuberances were referred to as apoptotic bodies by Kerr *et al.* (16) and are one of the primary diagnostic features used to identify apoptotic cells.

On day 16 of pupal–adult development, the ISMs of *M. sexta* are composed of large cylindrical fibers ≈5 mm long. The membrane displays some longitudinal furrows and regular arrays of sarcomeres (Fig. 1*C*). When the ISMs die after adult eclosion, the contractile proteins are rapidly degraded (14). The cells shrink but maintain the same length because they are attached to the segmental boundaries of the abdomen. This reduction in cell volume causes the membrane to bunch up and wrinkle (Fig. 1*D*). As the process continues, the membrane accordions to produce a morphology that is reminiscent of elephant knees. In no preparation did we observe the large spherical protuberances seen on the surface of D011.10 cells. Consequently, it appears that dying ISM cells do not produce apoptotic bodies.

Changes in Nuclear Ultrastructure Accompanying Cell Death. Next we examined the nuclear ultrastructure of normal and dying D011.10 cells and ISMs (Fig. 2). Untreated D011.10 cells have a small amount of granular cytoplasm and a large nucleus containing diffuse, lightly staining heterochromatin (Fig. 2*A*). When these cells are examined 16 hr after dexamethasone treatment, the nucleus is distorted due to the formation of the apoptotic bodies (Fig. 2*B*). In these cells, the chromatin is very electron dense and condenses

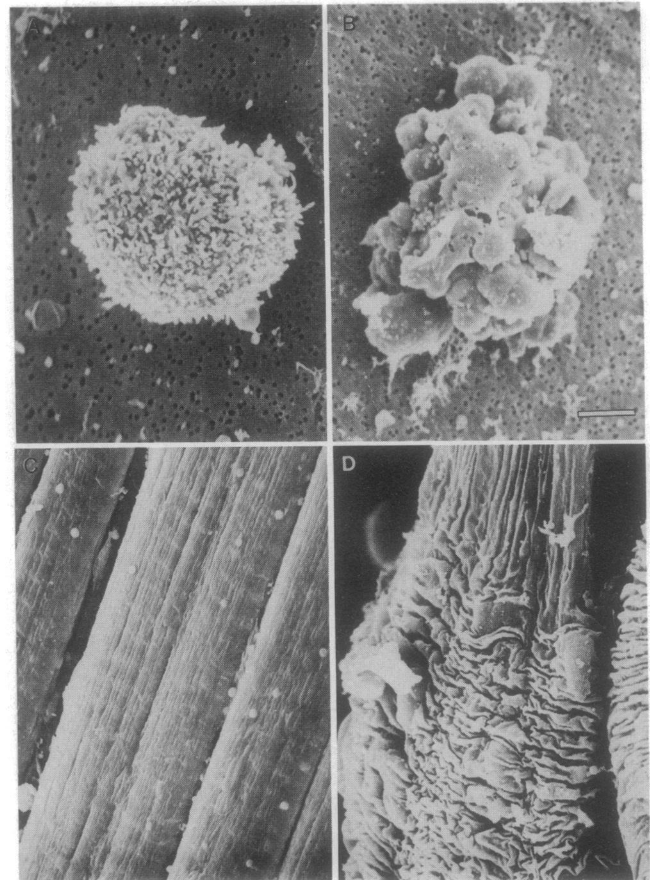

FIG. 1. Scanning electron micrographs of normal and degenerating mouse D011.10 T-cell hybridoma cells and moth ISMs. (*A*) Control D011.10 cells *in vitro*. (*B*) D011.10 cells were induced to die by 62.5 μM dexamethasone and were examined 17 hr after treatment. (*C*) ISM fibers fixed at length from a pupa on day 16 of development (48 hr before eclosion). (*D*) ISM fiber from an adult moth (17 hr after eclosion). [Bar = 2.3 μm (*A*), 2.8 μm (*B*), 10.5 μm (*C*), and 4.2 μm (*D*).]

along the inner surface of the nuclear envelope. Identical results were seen with dexamethasone-treated thymocytes *in vivo* (ref. 12; S.W.S. and B.A.O., unpublished work). These changes in nuclear ultrastructure are diagnostic of apoptosis (16).

In contrast, the nuclei of day 16 ISMs are multi-lobed, elongated structures with small amounts of dispersed, lightly staining heterochromatin (Fig. 2*C*). During ISM death, the nuclei round up, and the nuclear membrane swells (Fig. 2*D*). The chromatin becomes pyknotic, as evidenced by focal condensations. However, this pyknosis does not involve dramatic dispersion and deposition of electron-dense chromatin along the inner surface of the nuclear membrane. These nuclei would not be classified as apoptotic by the criteria of Kerr *et al.* (16).

Organization of Chromosomal DNA. The changes seen in nuclear morphology that accompany apoptosis coincide with alterations in the organization of chromosomal DNA (23). An endogenous nuclease cleaves the DNA between individual nucleosomes, producing degraded fragments that differ in size by ≈180 bp (12, 24). This fragmented chromosomal DNA can be visualized by isolating genomic DNA, subjecting it to electrophoretic size fractionation in agarose, and staining it with the fluorescent intercalating dye ethidium bromide. Thymocyte DNA from control- or saline-injected mice is high-M_r DNA and remains at the top of the gel (Fig. 3*A*, 0 hr). However, within 4 hr after dexamethasone injection, the

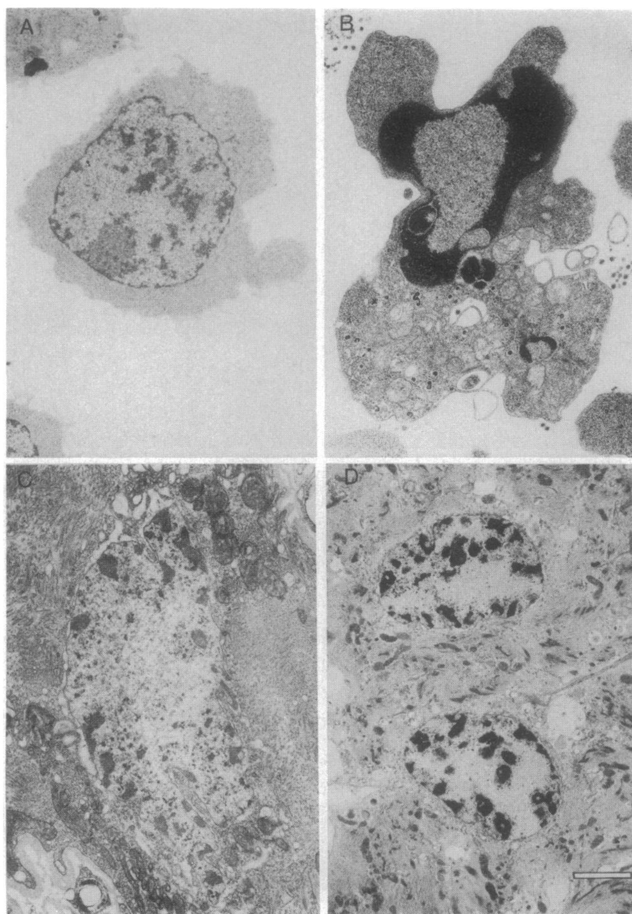

FIG. 2. Transmission electron micrographs of normal and dying D011.10 cells and moth ISMs. (*A*) Control D011.10 cells. (*B*) D011.10 cells induced to die by 62.5 μM dexamethasone and examined 16 hr after treatment. (*C*) ISM nucleus from day 16 of development (48 hr before eclosion). (*D*) Nuclei of degenerating ISM cell (18 hr after eclosion). [Bar = 2.2 μm (*A*), 1.7 μm (*B*), 1.6 μm (*C*), and 2.2 μm (*D*).]

concentration of high-M_r DNA is reduced, and a nucleosomal ladder of fragments is produced (Fig. 3*A*). The DNA continues to be degraded during the cell death period, and the accumulation of fragments is very dramatic 18 hr after steroid treatment.

One virtue of the ISMs as a model system for studying PCD is that large amounts of pure, synchronously dying cells can be isolated for biochemical analysis (20, 25). To examine the organization of ISM DNA, muscles were isolated at various developmental stages before and after the onset of degeneration. ISMs were also obtained from insects injected with the steroid 20-hydroxyecdysone on day 17 of development because this treatment prevents the death of ISMs on day 18, as normally occurs (15). After extraction, equal amounts of ISM DNA from each stage were fractionated by size in agarose and stained with ethidium bromide. All the samples contain high-molecular-size DNA with no evidence of degradation (Fig. 3*B*). Even at 24 hr posteclosion, when the contractile proteins are completely degraded and the ISMs are composed primarily of internalized membranes and adhering nuclei, the DNA is still high-molecular-size DNA (data not shown). To rule out the possibility that some small percentage of the DNA was degraded into a nucleosomal ladder but was below the level of detection by ethidium bromide, the DNA was transferred to a membrane and probed with ^{32}P-labeled *Manduca* genomic DNA (Fig. 3*C*). The blotting method used reduces the transfer of high-molecular size DNA in favor of lower-molecular-size species. Although some sheared DNA was present in several of the samples, presumably due to damage during isolation, there is no evidence of any nucleosomal ladder.

Expression of Ubiquitin. As mentioned above, the death of both immature T cells and the ISMs requires *de novo* expression of specific sets of genes. For example, treatment with the transcriptional inhibitor actinomycin D blocks cell death in the ISMs (20) and T cells (ref. 19; S.W.S. and B.A.O., unpublished work). Using a differential hybridization approach, cDNA clones were isolated for four genes from *Manduca* that are up-regulated during ISM cell death (20). One of these clones was shown to be the product of the polyubiquitin gene (25).

The ISMs become committed to die early on day 18 of pupal–adult development and actually begin the degeneration process coincident with eclosion late on day 18 (15). The ISMs were removed from animals at several times during days 17 and 18. RNA was isolated, fractionated by size in agarose, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled mouse polyubiquitin cDNA clone (22). As shown in the Northern blot of Fig. 4*A*, the abundance of polyubiquitin mRNA is very low on day 17 but accumulates to high levels when the muscles become committed to die on day 18. At that point, several high-molecular-size polyubiquitin transcripts are detected. If ISM cell death is delayed with 20-hydroxyecdysone, accumulation of these transcripts is very much reduced. Re-probing the blot with a constitutively expressed sequence verified that amounts of RNA in each lane were equal (data not shown).

We sought to determine whether the polyubiquitin gene was also up-regulated during T-cell death. Mice were injected with dexamethasone or saline, and the thymocyte RNA was probed as above on a Northern blot. Two distinct hybridizing transcripts appeared at every stage examined. Induction of T-cell death with dexamethasone did not detectably alter the levels of these transcripts (Fig. 4*B*). In separate experiments, we have induced cell death with either dexamethasone or the anti-T-cell receptor antibody F23.1 in D011.10 cells. These treatments did not alter the levels of polyubiquitin mRNA (S.W.S., L.M.S., and B.A.O., unpublished work).

DISCUSSION

The programmed deaths of both mouse T cells and moth ISMs share two fundamental features. Both are initiated by specific physiological signals and both require *de novo* gene expression. However, once the cells begin to die, they do so in seemingly different ways. The T cells die by apoptosis, which is characterized by membrane blebbing, chromatin margination, and the breakdown of chromosomal DNA into nucleosome-sized fragments. None of these features are seen in the ISMs. Instead, these cells exhibit membrane wrinkling, nuclear pyknosis, and the retention of high-M_r genomic DNA.

There are two possible explanations for these results. (*i*) Apoptosis includes a variety of different steps, and the T cells and ISMs represent different ends of this spectrum. (*ii*) The ISMs do not undergo apoptosis but, rather, die by a different molecular mechanism. Because we were unable to detect any of the known morphological or biochemical events associated with apoptosis in the ISMs, we favor the latter hypothesis.

Were the ISMs dying by a nonapoptotic cell death program, they might not be the only cells to do so. Data from the literature suggest that a number of other cells die in a manner inconsistent with apoptosis. In addition to the ISMs, there are reports of several other dying cells that fail to generate chromosomal DNA ladders during the death process. These cells include glucocorticoid-treated hippocampal neurons (26), nerve growth factor-deprived neuronal PC-12 cells (27),

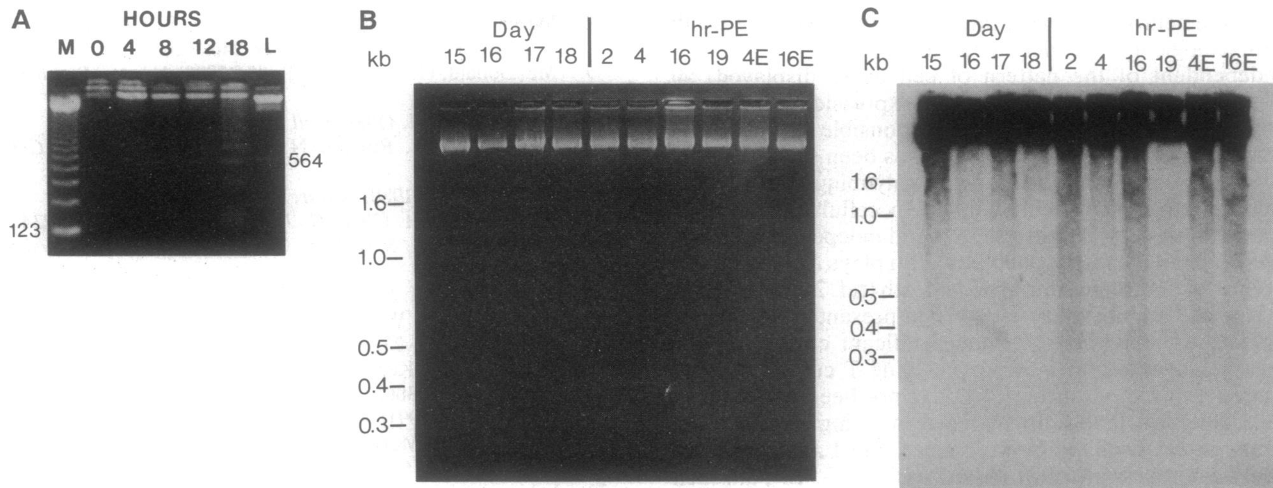

FIG. 3. Changes in size of chromosomal DNA during PCD in T cells and ISMs. (*A*) Generation of nucleosomal ladder from thymocytes of dexamethasone-treated mice. Times reflect hours after injection, whereas the 0 hr control lane represents DNA from an uninjected animal. DNA (2.5 μg) from each sample was fractionated in 2% agarose and stained with ethidium bromide (10 μg/ml). The molecular size standard M is a 123-bp repeat, and the L lane contains *Hin*dIII-digested λ phage DNA as a molecular size marker. (*B*) Retention of high-molecular-size DNA in degenerating ISMs. Five micrograms of ISM DNA was fractionated, as described above. d, Day of adult development; hr-PE, hours posteclosion. DNA samples labeled E are from animals treated on day 17 of development with 20-hydroxyecdysone from which the ISMs were removed 4 and 16 hr after the normal time of adult eclosion. (*C*) Southern blot of ISM genomic DNA, showing the retention of high-molecular-size DNA during cell death. DNA from the above gel was transferred to a membrane and hybridized with ^{32}P-labeled *Eco*RI-digested *Manduca* DNA under stringent conditions.

trophic factor-deprived oligodendrocytes (28), embryonic chicken spinal motor neurons (S. E. McKay, L.M.S., and R. Oppenheim, unpublished work), and metamorphosing insect salivary glands (29).

There are some cases where cells may undergo apoptosis, but DNA fragments cannot be detected. This situation can arise when dying cells represent only a small percentage of total tissue mass. In such instances, an examination of the nuclear ultrastructure can facilitate characterization of the cell death mechanism. As described above, DNA degradation correlates with the dispersion of chromatin along the nuclear envelope (23). Many dying neurons display an apoptotic morphology, which has been referred to as type I

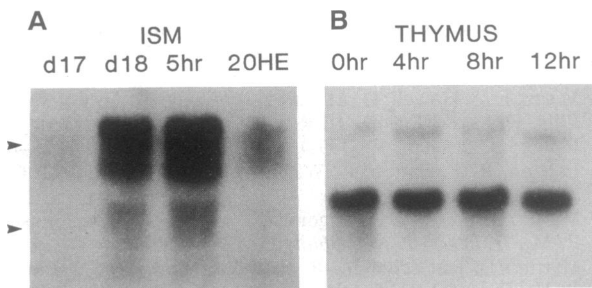

FIG. 4. Patterns of polyubiquitin mRNA accumulation. (*A*) Northern blot of *Manduca* ISM RNA probed with a ^{32}P-labeled polyubiquitin cDNA. The ISMs were removed for RNA isolation from animals on day 17 (d17), day 18 (d18), 5 hr posteclosion (5 hr), and late on day 18 after treatment with 20-hydroxyecdysone (20HE). After fractionation and transfer, the RNA was hybridized with a ^{32}P-labeled cDNA clone (3JSF) encoding the mouse polyubiquitin gene (22). Identical results were obtained with a *Manduca* polyubiquitin cDNA (25). Re-probing the blot with the constitutively expressed 15-3 gene (20) showed equal loading of RNA (data not shown). Arrows indicate molecular size of transcripts (4.3 and 1.7 kb). (*B*) Mouse thymocyte Northern blot probed with a ^{32}P-labeled mouse polyubiquitin cDNA clone (22). Thymocyte RNA was isolated at the times indicated after dexamethasone injection. Northern blots were treated as described above. Re-probing the blot with the constitutively expressed glyceraldehyde 3-phosphate dehydrogenase gene showed equal loading of RNA (data not shown). Arrows indicate the same molecular sizes shown in Fig. 3*A*.

degeneration by neuroanatomists (ref. 30; for review, see ref. 31). However, other studies have documented that certain dying neurons do not exhibit chromatin margination and possess a nuclear morphology comparable to that seen in the ISMs. This pattern has been referred to as type II degeneration or autophagy (30, 31) and includes Rohon–Beard cells (32), frog spinal motor neurons (33), and isthmo–optic nucleus neurons (34). Further evidence that type I and II degenerations do, in fact, reflect distinct cell death programs is provided by studies of the ciliary ganglion. During normal PCD, these cells undergo type II (nonapoptotic) cell death, whereas denervation-induced death proceeds via type I (apoptotic) cell death (35). Therefore, the same cell can apparently activate two distinct cell death programs, depending on the triggering stimulus.

In examining the data present in the literature, apoptosis appears to be rare in invertebrates (34). In fact, the only demonstration of invertebrate cells generating DNA ladders are lepidopteran tissue culture cells infected with a specific bacculovirus (36). In the latter case, whether the laddering is an endogenous property of the cell line or a response to a virally introduced gene is unclear. In fact, apoptosis has been suggested to have evolved as an antiviral defense (37). Consequently, apoptosis might be a phylogenetically newer cell death mechanism. If this hypothesis is true, it is useful to speculate about the selective pressures responsible for the appearance of such a mechanism. The rapid breakdown of DNA seen in apoptosis ensures that even though a targeted cell does not immediately die, it is mitotically incapacitated. This feature would be invaluable for ensuring the efficient removal of dangerous mitotic cells, such as self-reactive T cells. In contrast, many other targeted cells appear to be postmitotic, and their inappropriate retention is relatively benign. Evidence for this comes from nematodes, where mutations in the *ced* (*ce*ll *d*eath) genes can block the death of all the cells normally fated to die (1, 38). Animals with these *ced* mutations are viable and appear to behave almost normally, despite a substantial increase in the number of neurons. At present it is not known whether cell death in *Caenorhabditis elegans* occurs via apoptosis because large amounts of synchronously dying cells cannot be obtained for analysis (1). Similar observations have been made in chick-

ens, where neurons normally fated to die can be rescued with pharmacological or surgical manipulations (6).

Independent of the pattern of cell death displayed, all PCDs appear to require *de novo* gene expression (for review, see ref. 2). At present, the genes responsible for mediating PCD are largely unknown. One that has been shown to play a role in the death of the ISMs is the polyubiquitin gene (25). Ubiquitin can be covalently linked to cellular proteins to mark them for degradation (39). Several independent lines of evidence strongly suggest that ubiquitin plays a major role in the removal of proteins during ISM death (ref. 24; A. L. Haas and L.M.S., unpublished work). Data presented here, however, suggest that there are not significant changes in the levels of polyubiquitin message in dying T cells. Although ubiquitin expression during PCD has not been surveyed in detail, ubiquitin levels do increase in dying muscles and neurons in *Drosophila* (J. W. Truman and L.M.S., unpublished work), neurons of *Manduca* (ref. 25; S. E. Fahrbach and L.M.S., unpublished work), tunicate stomach (40), and interdigital cells of chicken embryos (S. E. McKay, L.M.S., and R. Oppenheim, unpublished work). Increases in ubiquitin were not detected during the death of dorsal root ganglia (41), cells thought to die via apoptosis (42). At present whether other apoptotic cells alter their patterns of ubiquitin expression or utilization is unknown.

In summary, the data presented here support the hypothesis that more than one pathway for mediating PCD may exist. Clearly apoptosis has been well characterized and shown to be a major mediator of PCD. However, perhaps use of the terms apoptosis and PCD interchangeably is inappropriate. With the isolation and characterization of putative cell death genes, hopefully a determination of how many different cell death mechanisms are used during animal development will be possible.

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