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Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*

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SUMMARY

We have studied the embryonic development of *Drosophila* hemocytes and their conversion into macrophages. Hemocytes derive exclusively from the mesoderm of the head and disperse along several invariant migratory paths throughout the embryo. The origin of hemocytes from the head mesoderm is further supported by the finding that in *Bicaudal D*, a mutation that lacks all head structures, and in *twist snail* double mutants, where no mesoderm develops, hemocytes do not form. All embryonic hemocytes behave like a homogenous population with respect to their potential for phagocytosis. Thus, in the wild type, about 80-90% of hemocytes become macrophages during late development. In mutations with an increased amount of cell death (*knirps*; *stardust*; *fork head*), this figure approaches 100%. In contrast, in these mutations, the absolute number of hemocytes does not differ from that in wild type, indicating that cell death does not 'induce' the formation of hemocytes. Finally, we show that, in the *Drosophila* embryo, apoptosis can occur independently of macrophages, since mutations lacking macrophages (*Bicaudal D*; *twist snail* double mutants; *torso*^{4021}) show abundant cell death.

Key words: hemocytes, macrophages, cell death, *Drosophila*

INTRODUCTION

Programmed cell death, or apoptosis, is a widespread phenomenon occurring during normal development of all metazoans. (Glücksmann, 1950; Saunders, 1966; Wyllie et al, 1980; Ellis et al., 1991; Raff, 1992). Experimental evidence indicates that cell death can be triggered by both intrinsic and extrinsic cues. The best studied example for intrinsically (cell-automomously) controlled cell death is the nematode *Caenorhabditis elegans.* Here, an invariant set of cells was defined which already at the time of their birth are 'doomed' to degenerate (for review, see Ellis et al., 1991). In contrast, in both vertebrate and invertebrate systems, numerous extrinsic (non cell-autonomously acting) factors that trigger cell death have been identified. Glucocorticoids stimulate death of thymocytes (Wyllie, 1980); Muellerian Inhibiting Substance (MIS) is responsible for cell death of the Muellerian ducts in male development (e.g., Tran et al., 1977); low levels of ecdysteroids initiate histolysis of neurons and muscle fibres in metamorphosing insects (Schwartz and Truman, 1984). For both vertebrate and invertebrate systems, genes have been defined that control the cell death program (for recent review, see Ellis et al., 1991; Vaux, 1993).

Cells that have undergone apoptosis are engulfed by macrophages, although other cells (e.g., epidermis) are also able to engulf their apoptotic neighbors (e.g. Wolff and Ready, 1991, for the *Drosophila* eye). Beside their role as 'scavengers', macrophages could also be more actively involved in the initiation of cell death. It had been proposed a long time ago that during metamorphosis of holometabolous insects, phagocytic cells actively trigger histolysis of larval tissues (e.g., Perez, 1910). This hypothesis was refuted in view of the fact that, in both the nervous and the muscular system of insect pupae, apoptosis begins in the absence of macrophages (e.g., Crossley, 1964). In contrast, the recent finding of Lang and Bishop (1993) that killing macrophages in developing mice by expressing diphteria toxin prevents cell death in the pupillary membrane and hyaloid vasculature of the eye suggests that macrophages are actively involved in cell death in this system.

In this paper, we have addressed the question of how macrophages develop in the *Drosophila* embryo and what is their relationship towards cell death occurring in this organism. In insects, macrophages represent a subpopulation of blood cells, or hemocytes, which are contained within the hemolymph space. In adults and larvae, hemocytes are formed by specialized hemopoietic tissues associated with the aorta and heart (for review, see Hoffman et al., 1979). The embryonic origin of hemocytes has been studied only in a cursory manner for a number of different insect groups. Most authors agree that the median mesoderm, i.e., the narrow band of mesoderm overlying the ventral nerve cord, gives rise to the hemocytes (Mori, 1979). A similar origin was also stated for the Dipteran *Dacus* by Anderson (1963), although this author emphasizes that blood cells precursors derive predominantly from the mesoderm of the head and anterior part of the trunk. Using hemocyte-specific markers, we have followed the development of these cells and their differentiation into macrophages. Our findings show that hemocytes derive from the mesoderm of the head and migrate along several invariant paths throughout the embryo. In the wild type, the large

majority of hemocytes develop into phagocytic macrophages; in mutations with an increased amount of cell death [*knirps* (*kni*; Nüsslein-Volhard and Wieschaus, 1980); *stardust* (*sdt*; Tepass and Knust, 1993); *fork head* (*fkh*; Weigel et al., 1989)], the number of hemocytes turning into macrophages approaches 100%. Analysis of these mutations shows further that the absolute number of hemocytes is not increased compared to wild type, indicating that cell death does not 'induce' the formation of hemocytes. Finally, in contrast to the above cited vertebrate system in which macrophages seem to be required for the initiation of cell death (Lang and Bishop, 1993), we show that in the *Drosophila* embryo cell death occurs in the absence of macrophages. In mutations lacking macrophages [*Bicaudal D* (*BicD*; Nüsslein-Volhard et al., 1982); *twist* (*twi*) *snail* (*sna*) double mutants (Simpson, 1983; Grau et al., 1984; D. Gullberg and L. I. F., unpublished observations); *torso* ^{4021} (*tor*^{4021}; Klinger et al., 1988)], cell death is abundant.

MATERIAL AND METHODS

Fly stocks and egg collections

We used the strong *twi* allele *twi*^{HH07} (Nüsslein-Volhard et al., 1984), the strong *sna* allele *sna*$^{4.26}$ (Lindsley and Zimm, 1992), the strong *sdt* allele *sdt*^{EH} (Eberl and Hilliker, 1988), the dominant *torso* allele *tor*^{4021} (Klingler et al., 1988), the strong *fkh* allele *fkh*^{1} (Jürgens et al., 1984), the *BicD* allele *BicD*^{7134} (Wharton and Struhl, 1989) and Oregon R as wild-type stock. Flies were grown under standard conditions and crosses were performed at room temperature or at 25°C. Egg collections were made on yeasted apple juice agar plates. Embryonic stages are given according to Campos-Ortega and Hartenstein (1985).

Markers and immunohistochemistry

The following markers were used in this study: the enhancer-trap line B4-2-27 (Bier et al., 1989; Hartenstein and Jan, 1992) to label pharynx myoblasts derived from the head mesoderm; the enhancer-trap line E8-2-18 (Bier et al., 1989; Hartenstein and Jan, 1992) to label hemocytes. These enhancer-trap lines express β-galactosidase that was detected with a polyclonal anti-β-galactosidase antibody (Cappel; dilution 1:2000). The polyclonal anti-peroxidasin (anti-X; Abrams et al., 1993; Nelson et al., 1994) was used to label the hemocytes in wild type and all examined mutants. This antibody was diluted 200-fold. Antibody stainings and sections of stained embryos were done as described previously (Tepass and Knust, 1993).

Other histological techniques

Embryos for examination in the transmission electron microscope were prepared as described previously (Tepass and Hartenstein, 1994). Embryos for semi-thin sectioning were prepared in the same way. 2 μm sections were cut on an LKB Ultrotom V and stained with a toluidine blue/methylene blue/borate solution.

Application of bromodeoxyuridine (BrdU)

The base analogue BrdU, which is incorporated into replicating DNA, was applied by permeabilizing staged, dechorionated embryos with octane (Sigma) for 3 minutes and spreading them on BrdU containing Grace medium (1 mg/ml). Embryos were allowed to develop at 25°C for specific times, then collected, and fixed for 30 minutes in a mixture of 4% formaldehyde in PEMS(0.10M Pipes, 2 mM $MgSO_4$, 1 mM EGTA, pH 7.0) with heptane. Next they were devitellinized in methanol. After several washes in PBT, embryos were incubated for 35 minutes in 2 N HCl to denature the DNA. After this step they were washed for 30 minutes in several changes of PBT. The preparations were then incubated for 1 hour in PBT+N, followed by an overnight incubation in a monoclonal antibody against BrdU (Beckton-Dickinson) at a dilution of 1:50. For further steps of antibody labelling see Ashburner (1989).

RESULTS

Origin of hemocytes in the head mesoderm

The hemocytes can be first identified approximately 2 hours after gastrulation (late stage 10) as a discrete subpopulation of mesoderm cells located in the head of the embryo. This part of the mesoderm, which we will call procephalic mesoderm, forms two vertical plates overlying the procephalic neuroblasts. To label the hemocytes, two markers, the PlacZ construct E8-2-18 (Bier et al., 1989; Fig. 1D) and an antibody against the extracellular matrix component peroxidasin (anti-X; Abrams et al., 1993; Nelson et al., 1994), were used. These markers recognize the large majority, if not all of, the hemocytes throughout development. Thus, based on observations of whole mounts and sectioned material, all cells that can be classified as hemocytes structurally (scattered cells of round or irregular shape located in the hemocoel) express E8-2-18 and peroxidasin.

BrdU incorporation experiments show that the procephalic mesoderm forms a separate mitotic domain, which undergoes four divisions (cell cycles 14-17) during embryonic stages 8-11 (data not shown). After the final division, the majority of procephalic mesoderm cells (approximately 300 on either side of the embryo) are recognizable as hemocytes. Only the anteriormost portion of the procephalic mesoderm (about 50 on either side) does not give rise to hemocytes. Instead, as revealed by another enhancer trap line (B4-2-27), these cells represent the population of myoblasts of the pharyngeal musculature (Fig. 1E,F). In addition to the procephalic mesoderm, small groups of mesoderm cells in the lateral and midventral part of the gnathal segments become hemocytes (Fig. 1A,C). However, we cannot entirely rule out that these cells originate in the procephalic mesoderm from where they migrate posteriorly and ventrally into the gnathal segments.

We believe that the procephalic and gnathal mesoderm represents the only source of hemocytes found in the late embryo. Both E8-2-18 and anti-peroxidasin label about 700 cells in the head of the late stage 11 embryo. This number remains constant throughout embryogenesis. During late embryogenesis, the fat body starts expressing peroxidasin (Fig. 2J), as well as E8-2-18 (data not shown), at a low level. However, cells of the fat body form a coherent tissue, which does not significantly change in cell number and, at least during the embryonic period, is not likely to contribute to the scattered population of hemocytes.

Migration of hemocytes

At the beginning of germ band retraction (early stage 12), the hemocytes start spreading throughout the embryo (Figs 2, 3). Moving anteriorly and ventrally, they populate the clypeolabrum and gnathal buds. Posteriorly directed migration brings them into the tail end of the germ band, which is folded over the anterior part of the germ band during this stage and abuds the head region. A substantial portion of hemocytes remain in the dorsal head region. Germ band retraction carries the group of hemocytes that previously have entered the tail end poste-

riorly. In the following stages (13-14), hemocytes migrate from both ends of the embryo towards its middle. During this migration hemocytes follow four different routes:

(1) mid-ventrally between the ventral epidermis and the ventral nerve cord;

(2) between the dorsal surface of the ventral nerve cord and the mesoderm;

(3) along the dorsal boundary of the epidermal primordium;

(4) along the gut primordium.

By late stage 14 most parts of the embryo are rather evenly populated with hemocytes (Figs 2, 3). Dense clusters can be observed in the head, as well as around the foregut and hindgut.

Differentiation of hemocytes

In early stages of development, hemocytes ultrastructurally represent typical small, round mesoderm cells (Fig. 4A). According to the nomenclature proposed by Gupta (1985), these cells should be designated as prohemocytes. During their migration between stages 12 and 13, prohemocytes start differentiating, developing prominent endoplasmic reticula and forming long processes, which radiate out from the cell bodies in all directions (Fig. 4B,D). Cells that show these ultrastructural characteristics have been classified as plasmatocytes (Gupta, 1985). All hemocytes that we have examined in the late embryo show the characteristics of plasmatocytes. In the following, we will use the (more widely used) general term hemocyte for this cell type.

From stage 12 onward, many hemocytes, in particular those ones surrounding the brain and ventral nerve cord, show phagocytic activity. They contain one or more vacuoles filled with dark inclusions which represent shrunken cytoplasm and pycnotic nuclei of ingested cells that have undergone apoptotic cell death (Fig. 4B). Hemocytes containing ingested cells (or cell fragments) are thereby histologically defined as macrophages. We have analyzed the temporal sequence in which macrophages appear in whole mounts of anti-peroxidasin-stained embryos. During late stage 13, approximately half of the hemocytes show phagocytic activity (Fig. 3B). Most of these early macrophages are located around the CNS and in the head, where cell death is particularly abundant (Abrams et al., 1993; Younossi-Hartenstein et al., 1993). At stage 13 and 14, hemocytes located in the interior of the embryo around the esophagus, midgut and hindgut are still small and (at least on the light microscopic level) do not yet show the inclusions that would define them as macrophages. However, towards later stages, the fraction of hemocytes turned macrophages increases and, by the end of embryogenesis, around 90% of the anti-peroxidasin-labeled cells are macrophages (Fig. 3D).

From stage 13/14 onwards, hemocytes possess an extensive rough endoplasmatic reticulum that is often dilated into lacunae (Fig. 4D), indicating a high secretory activity that is typical for cells producing extracellular matrix (see Discussion). By contrast to other tissues in the late embryo and larva, the cell surface of hemocytes is not covered with a basement membrane (Fig. 4E,F).

The hemocyte-macrophage conversion depends on cell death

Most of the dead cells in the embryo are ingested and degraded by hemocytes. Only occasionally it can be observed that dead cells have been phagocytosed by other cells (e.g. epidermal cells; Fig. 4C). As described above, hemocytes convert into macrophages at different times during development. Thus, whereas most of the hemocytes located around the nervous system and in the head have ingested cellular debris by mid-embryogenesis, few of the hemocytes that surround the gut seem to have done so. Even later (stage 17), a certain fraction of these interiorly located hemocytes do not contain cellular debris. This could indicate that they represent a separate class

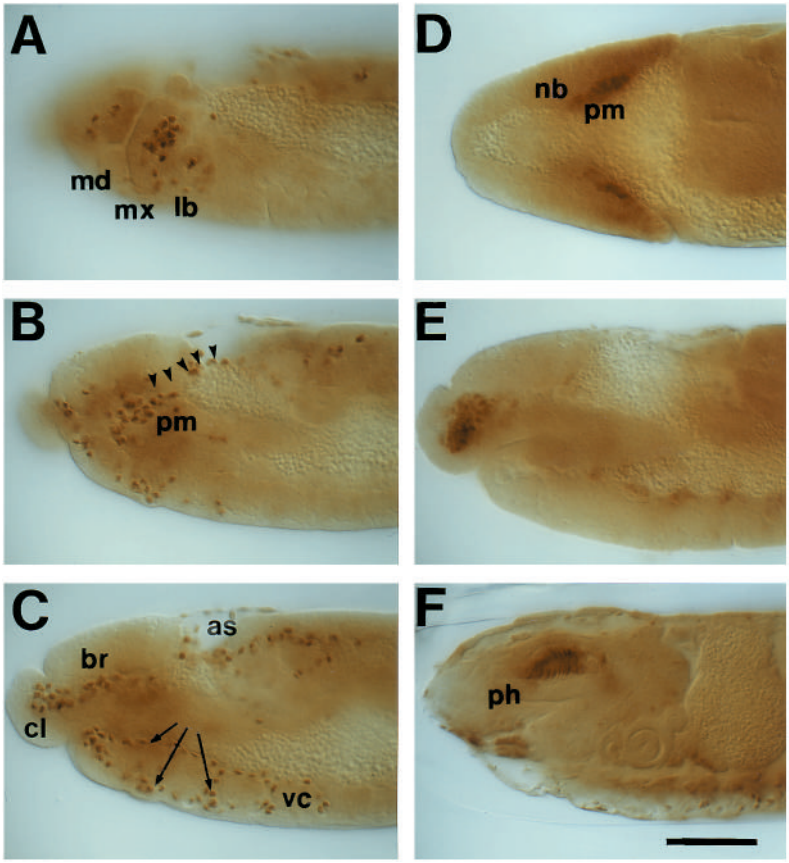

Fig. 1. Origin of hemocytes in the head mesoderm. (A-F) Whole mounts of embryos labeled with the enhancer detector lines E8-2-18 (A-D) expressed in hemocytes, and B4-2-27 (E,F), expressed on the pharynx muscles. (A-C) Three different focal planes (from superficial to deep) of a stage 11 embryo in lateral view. In this and all following figures, anterior is to the left, dorsal to the top. Early hemocytes (prohemocytes) appear in the mesoderm of the gnathal buds (lb, labium; md, mandible; mx, maxilla), the procephalon (pm), the clypeolabrum (cl), and the anterior portion of the median mesoderm [arrows in (C)]. Some hemocytes have already migrated beneath the amnioserosa (as) toward the tail end of the germ band (arrowheads in B mark migration route). (D) Ventral view of a stage 10 embryo, showing procephalic hemocytes prior to the onset of their migration. (E) Stage 11 embryo, lateral view, depicting the origin of the pharyngeal musculature in the mesoderm of the clypeolabrum. Distribution of pharyngeal muscles in a late embryo is shown in F. Other abbreviations: br, brain; nb, procephalic neuroblasts; ph, pharynx; vc, ventral nerve cord. Scale bar, 60 μm.

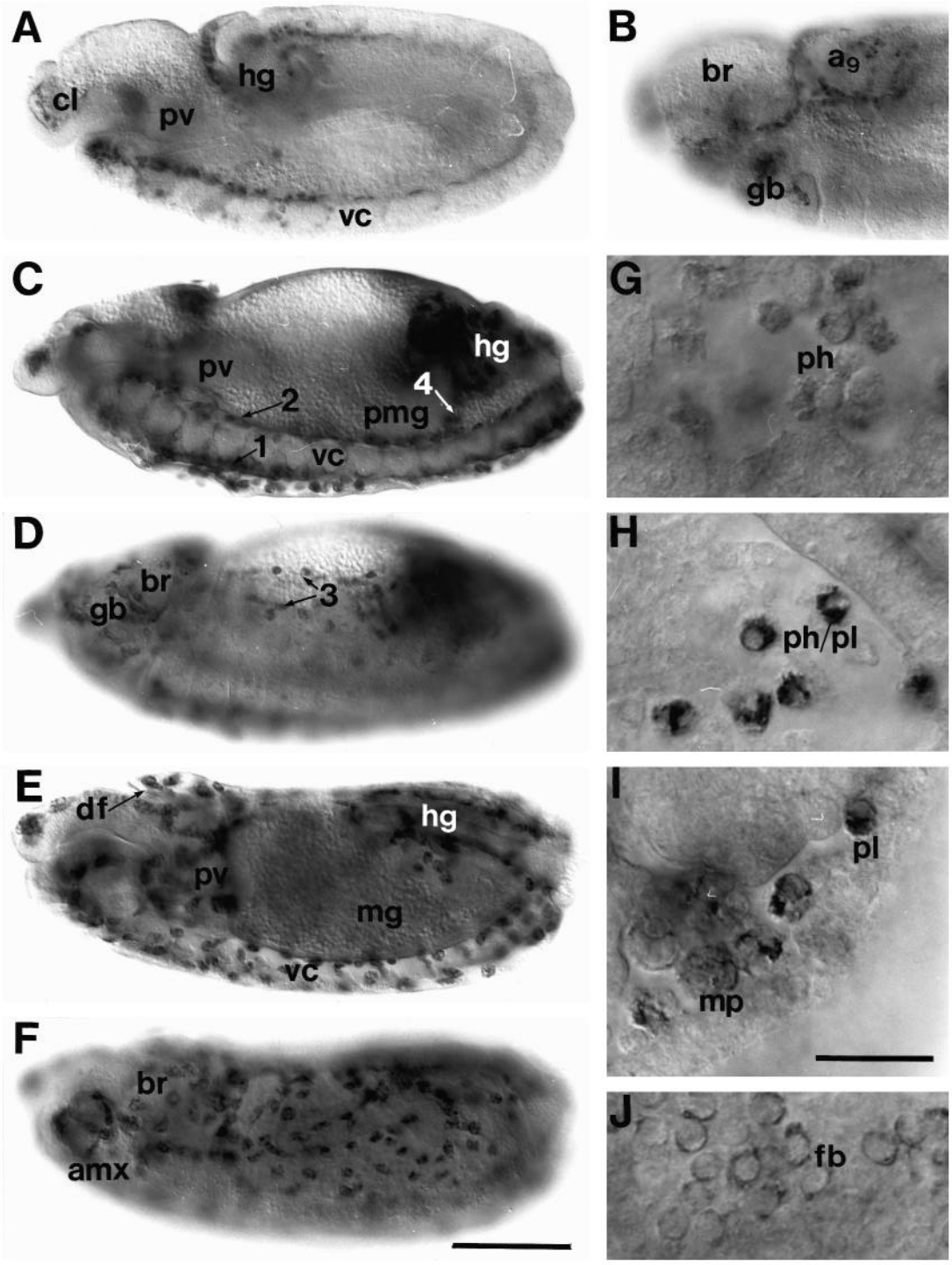

Fig. 2. Migration and differentiation of hemocytes. (A-F) Whole mounts of embryos (lateral view) at different stages stained with anti-peroxidasin antibody which labels hemocytes. (G-J) Magnified views of anti-peroxidasin-labeled hemocytes. During stage 11 (A, mid-sagittal plane of focus; (B, superficial plane of focus) early hemocytes (prohemocytes) mainly occupy the posterior region of the procephalon and the gnathal segments (gb, gnathal buds); in addition, they have invaded the clypeolabrum (cl) and the tail (a9, 9th abdominal segment) which, due to germ band elongation, lies adjacent to the head. (G) Close up view of prohemocytes (ph) which are typified by low level of peroxidasin-expression, small size and regular round shape. At stage 13 (C, mid-sagittal plane of focus; D, superficial plane of focus) the germband has retracted; the bulk of hemocytes still occupy the head and tail end of the embryo. They migrate anteriorly and posteriorly, respectively, along four different paths indicated by numbers (1, ventral surface of ventral nerve cord (vc); 2, dorsal surface of ventral nerve cord; 3, dorsal epidermis; 4, gut primordia). (H) Hemocytes of stage 13 embryo at high magnification. These cells express higher level of peroxidasin and have adopted a more irregular shape (typical for plasmatocytes; pl). At stage 16 (E, mid-sagittal plane of focus; F, superficial plane of focus), hemocytes are dispersed rather evenly throughout the embryo. Most superficially located hemocytes have become macrophages (mp), as seen at high magnification in panel I. By contrast, most hemocytes surrounding the proventriculus (pv) and hindgut (hg) have not yet started to phagocytose. (J) Low level of peroxidasin expression in the fat body (fb) of a stage 15 embryo. Other abbreviations: amx, antenno-maxillary complex; br, brain; df, dorsal fold; mg, midgut; pmg, posterior midgut rudiment. Scale bars: (A-F) 90 μm; (G-J) 30 μm.

of hemocytes, which do not possess phagocytic activity; alternatively, it could be the absence of cell death from the gut tissues that is responsible for the lower rate at which hemocytes located close to the gut convert into macrophages. According to this hypothesis, all hemocytes have the potential to phagocytose and thereby become macrophages once they come into contact with degenerating cells. Since cell death is indeed rare, if not absent, in the embryonic gut (Abrams et al., 1993; own observation), hemocytes surrounding this structure have no opportunity to phagocytose. To test this hypothesis, we analyzed the pattern of macrophages in *fkh* mutant embryos, in which most of the gut epithelium degenerates during stages

12/13 (Weigel et al., 1989). In these embryos, all interior hemocytes do become macrophages at an early stage (Fig. 5). In addition, the massive, locally circumscript cell death in the gut seems to attract hemocytes, since the proportion of interior versus superficial macrophages is significantly higher in *fkh* than in wild type. We conclude that all of the hemocytes stained by the anti-peroxidasin-antibody form a homogenous population with respect to their ability to become macrophages.

Hemocyte formation is independent of cell death

Hemocytes were counted in embryos carrying a mutation in the *sdt* or *kni* genes. In these mutants, the amount of cell death is dramatically increased. In *sdt*, most epithelial cells deriving from the ectoderm die during an extended period of time (Tepass and Knust, 1993). In *kni* mutant embryos, most cells located in the *kni* domain of expression (Nauber et al., 1988) die (own observation). Despite the high amount of cell death in both mutants, the number of anti-peroxidasin-positive cells is not significantly increased (Fig. 6). We counted (per half embryo) approximatly 345 anti-peroxidasin-positive cells for *sdt* (n=3; 320, 341, 373) and 320 for *kni* (n=2; 309, 331) versus 365 for wild type (n=4; 372, 389, 368, 331). These figures indicate that the number of hemocytes does not adapt to an increased need; the additional cell death does not 'induce' extra mesoderm cells to form hemocytes. To accomodate the large amount of cellular debris, macrophages in both mutations grow to a larger average size. In addition, cellular debris also becomes located extracellularly in the hemolymph.

Hemocytes cannot be recruited from the mesoderm of the trunk and tail

In embryos carrying the *BicD* mutation, head (including procephalic and gnathal mesoderm), thorax and anterior abdomen are absent. Instead, a second posterior abdomen with reversed polarity differentiates (Nüsslein-Volhard et al., 1982). We labeled *BicD* mutant embryos with anti-peroxidasin antibody to investigate whether, in the absence of the head mesoderm, cells of the trunk are able to give rise to hemocytes. In *BicD* mutant embryos hemocytes could not be detected (Fig. 7C,D). These observations suggest that, in the absence of head mesoderm, the remaining (trunk) population of mesoderm cells is unable to produce hemocytes.

Cell death occurs independently of macrophages

The analysis of the *BicD* phenotype, as well as two other mutations, *twi sna* double mutants and *tor*^{4021}, demonstrates that macrophages are not required for cell death to occur. Despite the absence of macrophages in late *BicD* mutant embryos, there is abundant cell death; cellular debris is located extracellularly in the hemolymph space (Fig. 7C,E). The same observation was made for *twi sna* double mutants (Fig. 7F) and *tor*^{4021} mutants (data not shown) which, like *BicD*, lack

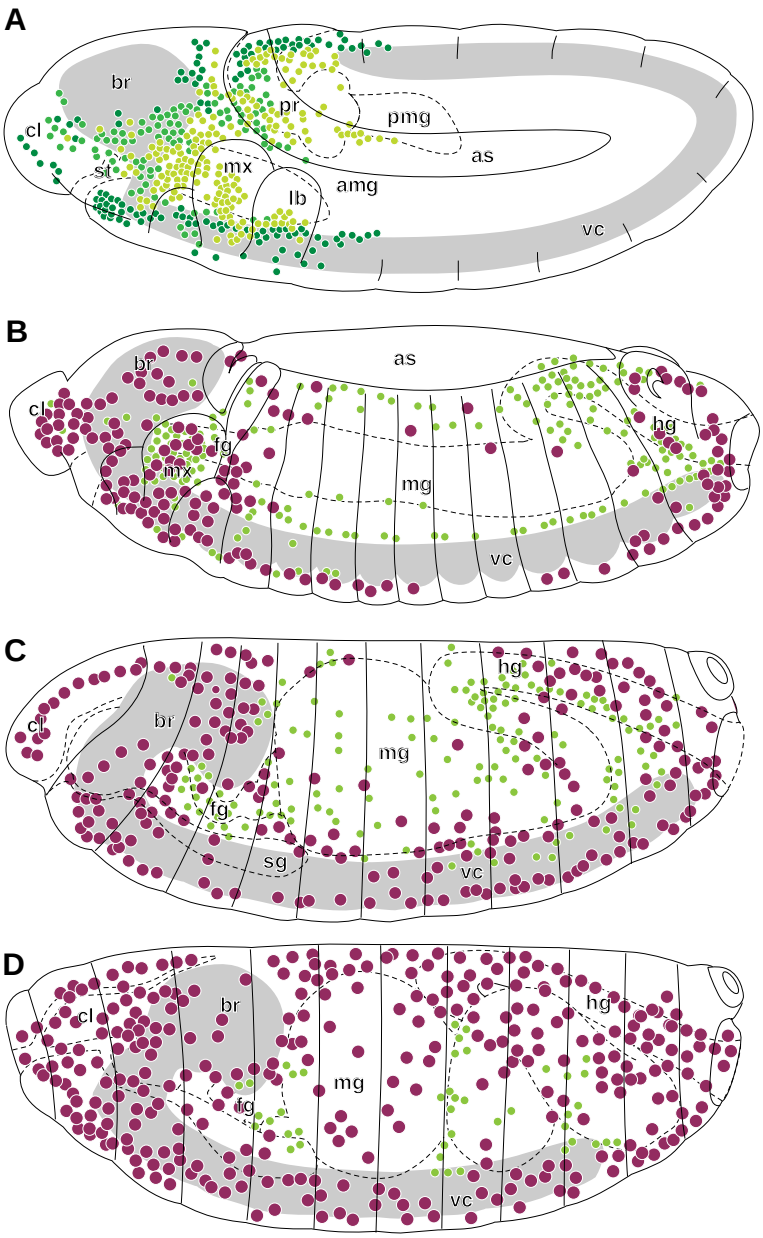

Fig. 3. Migration of hemocytes and conversion into macrophages. (A-D) Drawings of embryos (lateral view) at different stages. Profiles of interior structures are shaded (CNS) or given as dashed lines (gut primordia). The distribution of hemocytes (based on camera lucida drawings of anti-peroxidasin labeled whole mounts of embryos) is indicated by colored dots. Prohemocytes or hemocytes that do not show phagocytic activity are represented by green dots (one dot = one hemocyte; dark green dots: hemocytes beneath the epidermis; light green dots: hemocytes associated with the gut); phagocytic hemocytes (macrophages) are indicated by magenta colored dots. At stage 11 (A), the full complement of hemocytes is present, though none of them has started to phagocytose. (B) During stage 13, hemocytes spread out through the embryo (compare to Fig. 3). Most of the superficially located hemocytes (contacting the CNS and the epidermis) become macrophages. (C) At stage 15, hemocytes are evenly distributed. Still, many of the deeply located hemocytes (contacting the gut) do not contain phagocytosed cellular material. (D) Stage 17 embryo in which only a small minority of non-phagocytic hemocytes remains. Abbreviations: amg, anterior midgut rudiment; as, amnioserosa; br, brain; cl, clypeolabrum; hg, hindgut; lb, labial bud; mx, maxillary bud; pmg, posterior midgut rudiment; pr, proctodeum; pv, proventriculus; sg, salivary gland; st, stomodeum; vc, ventral nerve cord.

head mesoderm. In these mutants, massive cell death occurs, leading to extensive clusters of cell fragments which take up much of the hemolymph space. These findings show that macrophages do not play an active role in the initiation of at least the majority of apoptosis.

DISCUSSION

Embryonic origin of hemocytes

Since hemocytes are cells that are not stationary but move freely in the hemolymph, it is difficult in the absence of suitable markers to determine their exact origin in the embryo. This problem has resulted in some controversy; besides the median mesoderm, on which most authors agree as the site of hemocyte formation in other insects, the coelomic sacs (i.e., lateral mesoderm) and the subesophageal body were proposed as tissues giving rise to hemocytes. As discussed by Mori (1979), these reports were almost certainly the result of the erroneous assumption that hemocytes, because they are spatially associated with coelomic sacs and subesophageal body in late embryos, have to derive from these tissues.

The median mesoderm can be defined at a stage when the cells of the formerly monolayered mesoderm regroup and form multiple layers. Dorsally, the mesoderm forms metamerically reiterated 'sacs' with an inner layer (splanchnopleura or visceral mesoderm) and an outer layer (somatopleura or somatic mesoderm). The mid-ventral portion of the mesoderm remains thinner than the lateral mesoderm and is defined as the median mesoderm. According to previous histological studies, part of the median mesoderm cells dissociate and become hemocytes (reviewed in Mori, 1979).

In *Drosophila*, the median mesoderm is a 1- to 2-cell-thick layer overlying the primordium of the ventral nerve cord during stages 11-12 and differentiates later in somatic muscle and fat body cells (Hartenstein and Jan, 1992). The present study shows that, in *Drosophila*, if at all, only the most anterior (gnathal) part of the median mesoderm gives rise to hemocytes. In fact it is possible that the hemocytes derive only from the procephalic mesoderm and associate with the median mesoderm of the gnathal segments during an early phase of their migration.

Classification and morphological differentiation of hemocytes in the *Drosophila* embryo

There exists a confusingly diverse nomenclature of insect hemocytes. The widely used classification of Gupta (1985) distinguishes between 7 classes of hemocytes. Of these, 2 classes have been described for the *Drosophila* larva (Rizki, 1978): the plasmatocytes and oenocytoids (Rizki calls the latter 'crystal cells'). Plasmatocytes are large, variably shaped cells which show phagocytic activity. Oenocytoids, in contrast, do not phagocytose; they are structurally defined by inclusions of fibrous or crystalline material (Gupta, 1985). We could not detect oenocytoids in the embryo. Furthermore, at least in the background of mutations with increased cell death, all hemocytes showed phagocytic activity, which is absent from oenocytoids. These findings indicate that oenocytoids may originate shortly before hatching (late stage 17) or during postembryonic development.

The hemocytes in the *Drosophila* embryo, according to the

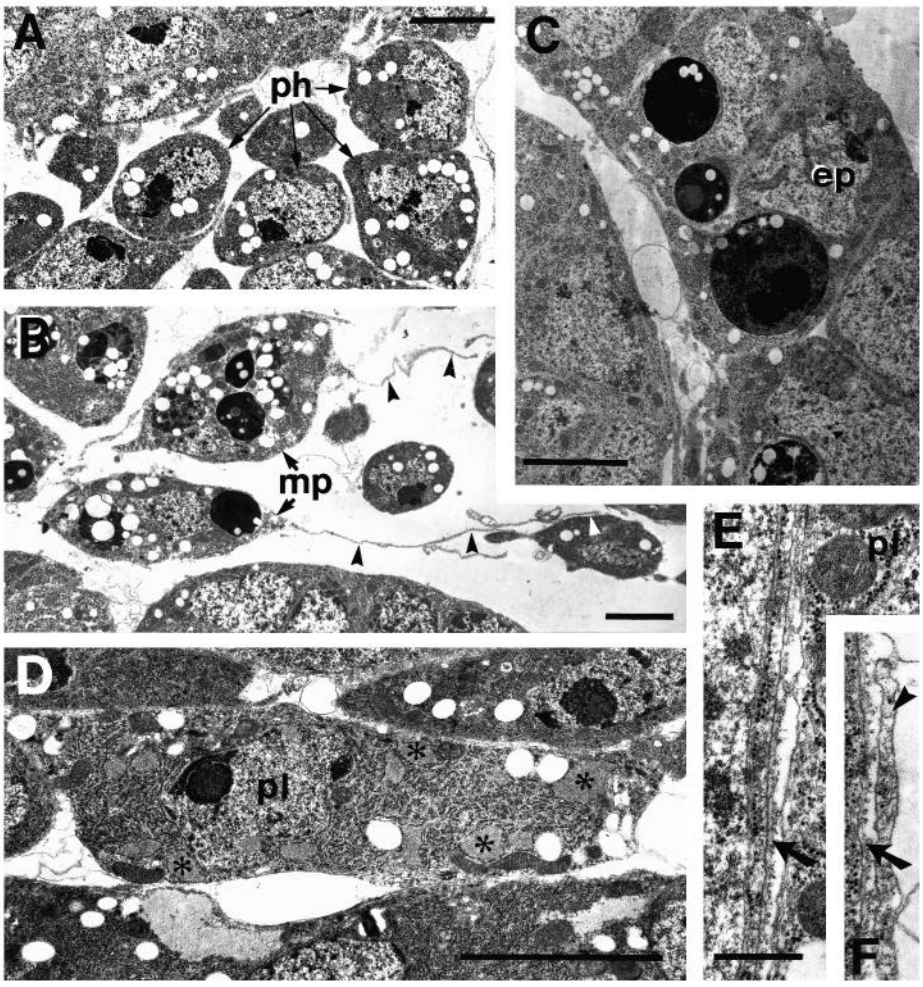

Fig. 4. Ultrastructural differentiation of hemocytes (A,B,D,E) and phagocytosis of apoptotic cells by the epidermis (C). (A) A group of prohemocytes (ph) in a stage 13 embryo. (B) Macrophages (mp) in a stage 14 embryo. Macrophages exhibit internal vesicles containing apoptotic (dark) bodies and extensive filipodia (arrowheads) that apparently 'probe' the enviroment. (C) Also the epidermal cells (ep; stage 13) are capable of engulfing dead cells (dark apoptotic bodies). (D-F) A hemocyte (plasmatocyte; pl) at stage 17. (D) The rough endoplasmic reticulum fills up most of the cytoplasm and has formed several large lacunae (asterisks) indicating an intense synthetic activity. In magnifications of the cell bodies (E) and a filipodium (arrowhead in F) of a hemocyte it can be seen that the hemocyte is contacting the basement membrane (arrows) that covers the surface of an adjacent cell. By contrast to all other cell surfaces that contact the hemolymph space, hemocytes do not possess a basement membrane. Scale bars: (A-D) 3 μm; (E,F) 330 nm.

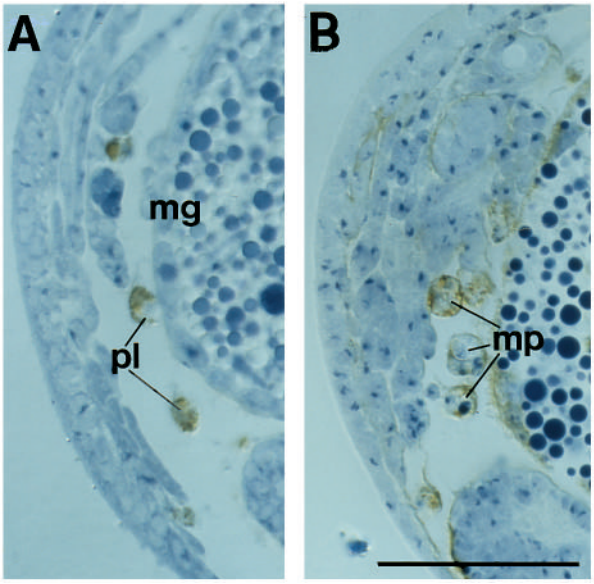

Fig. 5. Hemocytes in *fkh*, a mutation in which the gut epithelium degenerates. (A,B) Parts of sections of stage 16 embryos (A, wild type; B, *fkh*) stained with anti-peroxidasin antibody. The midgut epithelium (mg) is absent in *fkh*, in which anti-peroxidasin-positive hemocytes have become macrophages macrophages (mp). In wild type, by contrast, hemocytes (plasmatocytes; pl) surrounding the intact midgut have not yet become phagocytic. Scale bar: 45 μm.

morphological criteria given by Gupta (1985), are plasmatocytes. In wild-type embryos most of the hemocytes have phagocytosed dead cell or cell fragments and are therefore operationally defined as macrophages. If cell death is elevated, the number of hemocytes that have incorporated cellular debris approaches 100%. These observations suggest that the hemocytes of the *Drosophila* embryo are a homogeneous group of cells all of which have the ability to act as macrophages if required.

In the second half of embryogenesis, the bulk of extracellular matrix molecules are secreted (Fessler and Fessler, 1989) and basement membranes develop that cover all cell surfaces that are in contact with the hemolymph (Tepass and Hartenstein, 1994) with the exception of the cell surface of the hemocytes themselves. The hemocytes are apparently a major source of extracellular matrix molecules including papilin, laminin, collagen IV, glutactin and tiggrin (Fessler and Fessler, 1989, Kusche-Gullberg et. al., 1992, Fogerty et al., 1994). The ultrastructure of embryonic hemocytes, which is characterized by an extensive often dilated rough endoplasmatic reticulum suggesting a high secretory activity, is consistent with this conclusion.

Macrophages and cell death in the *Drosophila* embryo

A substantial fraction of cells die during normal embryonic development of *Drosophila*. An analysis of the pattern of cell death has recently been published (Abrams et al., 1993). Among the dying cells are mainly epidermal and neural precursors. Cell death is particularly abundant in the head during mid-embryogenesis. Here, contiguous regions of the head ectoderm degenerate, leading (among other mechanisms) to the drastic reorganization of the head structures during the head

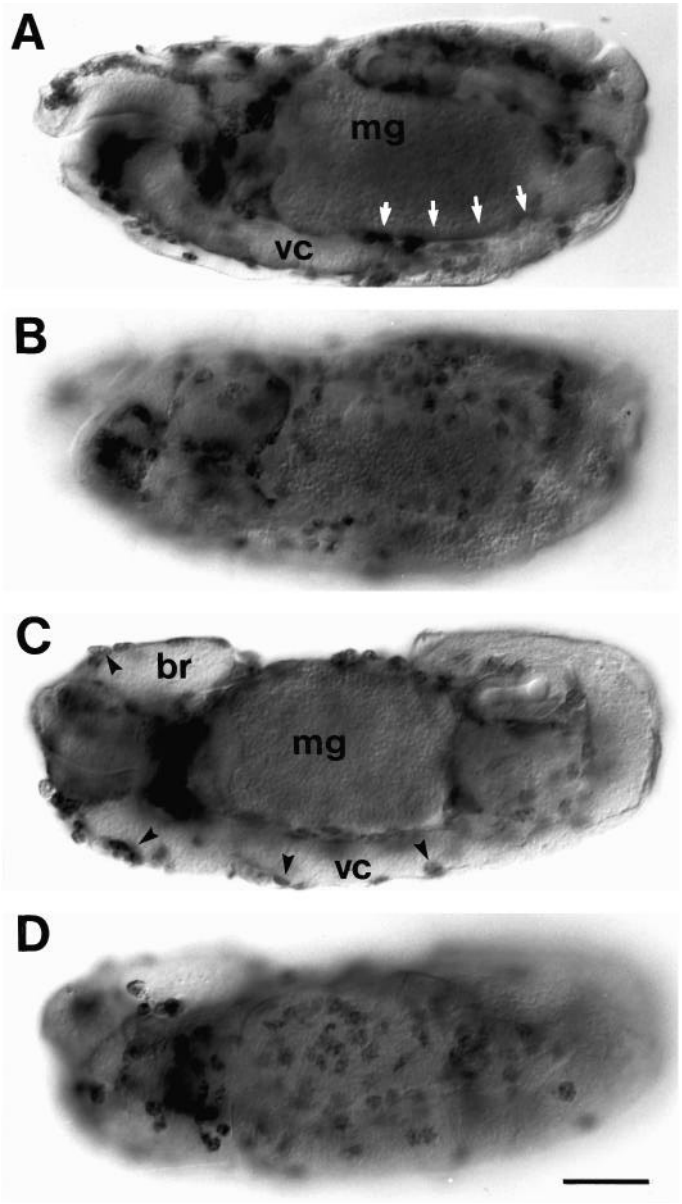

Fig. 6. Pattern of hemocytes in *kni* (A,B) and *sdt* (C,D), two mutations with increased cell death. All panels show whole mounts of stage 15/16 embryos (lateral view) stained with anti-peroxidasin antibody. (A,C) Midsagittal planes of focus; (B,D) superficial planes of focus. In *kni*, large portions of the abdominal CNS and epidermis degenerate (white arrows in A point at wide gap in ventral nerve cord). In *sdt*, most parts of the epidermis degenerate so that the CNS and gut is exposed at the surface. Arrowheads in C point at superficially exposed hemocytes. In both mutations, the total number and distribution of hemocytes corresponds to that of the wild type (compare this figure with Fig. 3E,F which shows wild-type pattern of hemocytes at a corresponding stage). Abbreviations: br, brain; mg, midgut; vc, ventral nerve cord. Scale bar: 60 μm

involution process (Younossi-Hartenstein et al., 1993). By contrast, no immediate consequence of the cell death occuring in the trunk has so far been detected. In all cases of cell death, most of the cellular debris is taken up by macrophages. In addition, similar to what has been described for the eye (Wolff

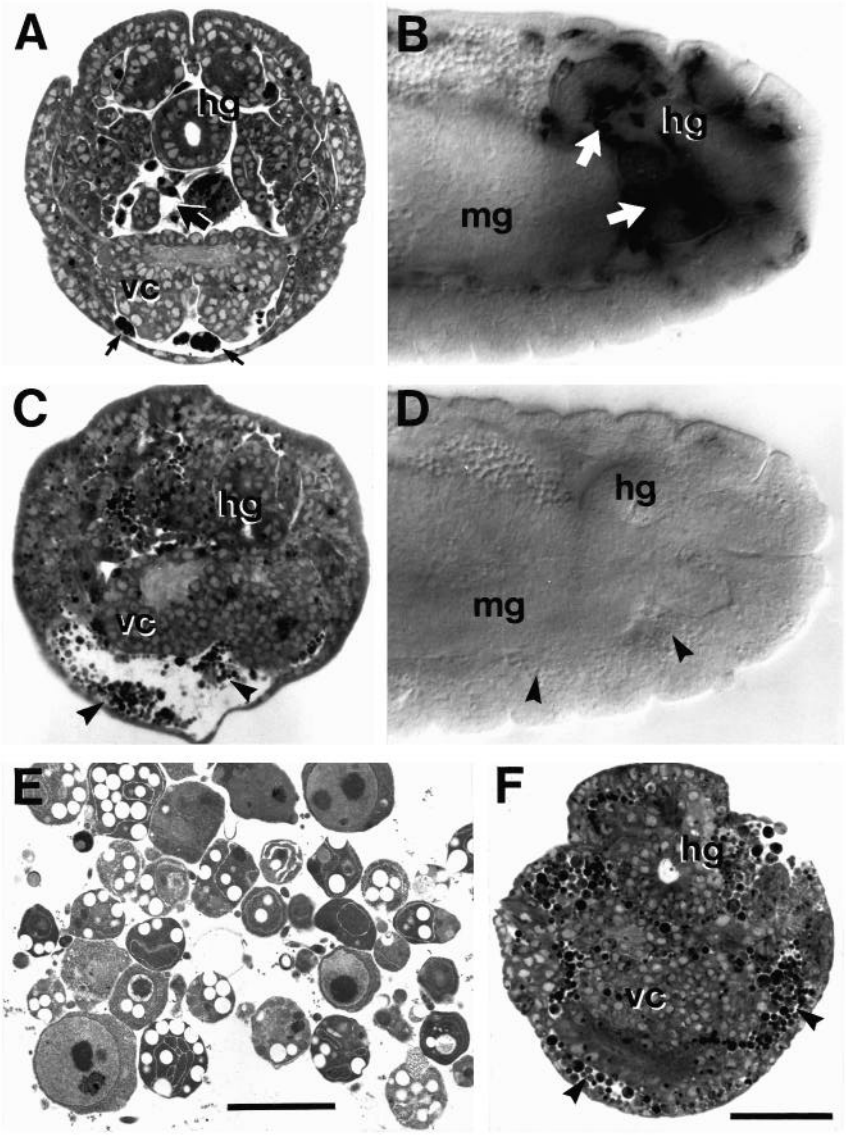

Fig. 7. Hemocytes are missing in a number of mutations without head mesoderm. (A,B) Stage 14 wild-type embryo (A, transverse section; B, dorsolateral view of posterior half of anti-peroxidasin-stained whole mount). Large arrows point at clusters of hemocytes surrounding hindgut (hg). Small arrows in A show large hemocytes containing phagocytosed cellular debris (dark dots). (C,D) *BicD* mutant embryos in which the head, including head mesoderm, are deleted and replaced by a mirror-image tail (C, transverse section; D, dorsolateral view of posterior half of anti-peroxidasin-stained wholemount). Anti-peroxidasin-labeled hemocytes are absent. Masses of fragments of degenerated cells have accumulated extracellularly (arrowheads). (E) An electron micrograph of accumulated apoptotic cells and cellular debris in the hemocoel of a late *BicD* mutant embryo. (F) A transverse sections of a *twi sna* double mutant embryo which lack mesoderm and, consequently, hemocytes. As pointed out for *BicD*, masses of cellular debris accumulate extracellularly (arrowheads). Abbreviations: hg, hindgut; mg, midgut; vc, ventral nerve cord. Scale bars: (A-D,F) 50 μm; (E) 3 μm.

and Ready, 1991), epidermal cells also occasionally phagocytose cellular debris (this study).

What is the relationship between hemocytes, macrophages and cell death in the *Drosophila* embryo? First, the formation of hemocytes from the head mesoderm is independent of cell death. Hemocytes are born before the onset of cell death. The number of mesoderm cells giving rise to hemocytes is intrinsically fixed and is not influenced by the amount of cell death, as evidenced by the fact that in mutants with dramatically increased cell death no addtional hemocytes are formed.

Secondly, the time and place at which hemocytes assume phagocytic activity seems to be determined by the pattern of cell death. In wild-type embryos, macrophages first appear in large numbers in the head and at the ventral aspect of the ventral nerve cord; by contrast, hemocytes located in the interior of the embryo around the gut become phagocytic late or not at all in the embryo. In the mutant *fkh*, in which the gut epithelium degenerates during stage 12/13 (Weigel et al., 1989), all deeply located hemocytes around the gut show phagocytic activity already at stage 13. Furthermore, the amount of cellular debris taken up by individual macrophages also depends on the extent of cell death. In mutants with increased cell death, macrophages have a larger average size and contain more vacuoles than in wild type. There seems to be a maximum volume of material a given macrophage can take up: in *kni* and *sdt*, a considerable amount of cellular debris remains extracellular, a phenomenon seen very rarely in wild type.

Finally, cell death is independent of macrophages. In three mutations that lack macrophages (*BicD*, tor^{4021}, *twi sna* double mutants), abundant programmed cell death could be observed. This result indicates that, as already stated for the cell death during metamorphosis (Wigglesworth, 1979), macrophages do not play any active part in at least the majority of cell undergoing cell death. The finding is in contrast to the recently published findings of Lang and Bishop (1993) who expressed diphteria-toxin under the control of the promoter for granulocyte/macrophage colony stimulating factor. This promoter specifically directs the expression in three subpopulations of macrophages, among them the hyalocytes found in the eye. Transgenic mice showed indeed degeneration of hyalocytes; in addition, two eye tissues, which normally are only transient,

the hyaloid vasculature and the pupillary membrane, persisted, suggesting that the hyalocytes are actively involved in their removal. In conclusion, the role of macrophages during the initiation of cell death may vary among different animal groups and/or organ systems. In order to resolve this issue, more needs to be learned about the molecular machinery that initiates and carries out the cell death program.

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