Fine Structural Aspects of the Anterior Necrotic Zone in the Leg Bud of the Chick Embryo

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Summary: A fine structural study was undertaken on the area called the anterior necrotic zone of leg buds of the chick embryo at stages 22–29. Physiologically dying cells, which were distributed in the mesenchymal area, were characterized by increased electron density of the cytoplasm, appearance of electron lucent vacuoles and condensed chromatin pattern. Many of the dying cells seemed to be fragmented into several pieces of cell debris during the necrotic process. Two kinds of cells were observed to take up the necrotic cells; one type, large cells 15 μm or more in size and irregular in shape, which had lysosomes, extended microvillus-like cytoplasmic projections and pseudopods, took up pieces of necrotic cells by phagocytosis and stored them in acid phosphate-negative and -positive vacuoles. The other type was several μm in diameter with numerous free ribosomes and stored a small number of vacuoles containing cell debris. At stage 29, profiles of necrotic cells lying free in the mesenchymal area, and cells containing one or two vacuoles filled with cell debris decreased in number as well as large cells containing numerous vacuoles, whose contents seemed to be degraded. When pieces of the anterior portion of chick leg buds at stage 24 were organ-cultured for 3 days after glutaraldehyde-fixed mouse red blood cells were injected, the red blood cells were taken up into cells in the mesenchymal area. Formation of large cells containing numerous vacuoles stuffed with red blood cells was rare. These findings may imply that most necrotic cells in the anterior necrotic zone are disposed of by macrophages and that some are endocytosed by mesenchymal cells.

It is well known that during development of vertebrate tissues, some cells spontaneously die in specific areas at specific stages. These phenomena are collectively called “physiological cell death” or “programmed cell death” in the sense that they are not pathological but programmed events in the course of development (for reviews of cell death, see Wyllie et al. 1980; Hinchiliffe 1981; Beaulaton and Lockshin 1982; Hurle 1988; Clarke 1990).

In limb buds of the chick embryo, features of physiological cell death are prominent during their development at early stages in three areas (the anterior necrotic zone, posterior necrotic zone and opaque patch) and, at later stages, in regressing interdigital tissue (Hinchiliffe and Johnson 1980; Hinchiliffe 1981; Hurle 1988). In the wing bud, Saunders et al. (1962) reported cell death observed in the posterior necrotic zone and suggested that cell death in this area is one of the chief contributors to the shaping of the forearm. Hurle and Hinchiliffe (1978) examined cell death taking place in the same area at the ultrastructural level including the detection of localization of acid phosphatase. In their observation, mesenchymal cells, which commence degeneration with acid phosphatase-positive autophagic vacuoles, are then fragmented and phagocytosed by neighboring mesenchymal cells. They suggested that mesenchymal cells taking up fragmented necrotic cells may transform into macrophages. However, it is necessary to further characterize the cells taking up and disposing of necrotic cells.

In the leg bud of the chick embryo, the anterior necrotic zone is exclusively and easily identified at stages 23 and 24 with Nile blue staining (Hinchiliffe and Johnson 1980; Hinchiliffe 1981), which is reported to vitally stain non-viable cells (Scott et al. 1977). Fine structural aspects must be studied also in this area.

The aim in the present report is to clarify at the ultrastructural level, 1) how physiologically dying cells are characterized in the anterior necrotic zone...
Materials and Methods

Fertilized chick eggs (strain, High-sex brown, purchased from Yamagishi, Hiroshima) were incubated at 38°C in a water-saturated incubator. Embryos were staged according to Hamburger and Hamilton (1951).

1) Nile blue stain

Chick embryos were stained with Nile blue solution similar to the method by Scott et al. (1977). In brief, Nile blue (Nile blue A, sulfate, Chroma) is dissolved and diluted to 1:20,000 in glucose-free Hanks' balanced salt solution (BSS). After the yolk sac and ovoalbumin were removed, chick embryos at stages 22-29 were put in the Nile blue solution and incubated for 30 minutes at 38°C. The specimens were photographed under a Nikon dissection microscope (SMZ-10 type).

2) Light microscopy

Chick embryos at stages 23 and 24 were fixed in Bouin's fluid, dehydrated in ethanol series and embedded in JB-4 embedding medium (Polysciences, Ltd.). Sections stained with hematoxylin-eosin were examined with a light microscope.

3) Semi-thin sections and ultrathin sections

Chick embryos at stages 22-29 were taken out in a glass petri dish in 0.1 M phosphate buffered saline and perfused through the heart ventricle with 2.5% glutaraldehyde solution buffered with 0.1 M Millonig's phosphate buffer (pH 7.4). For perfusion fixation, a glass capillary connected with a polyethylene tube was used. Anterior portions of leg buds were cut into small pieces, immersed in the same fixative for 2 hours at 4°C, and post-fixed in 1% OsO₄ with 0.1 M Millonig's phosphate buffer containing 5% sucrose for 1 hour at 4°C. After being rinsed in 10% sucrose solution 5 times, the tissue pieces were stained en bloc in 3% uranyl acetate solution for 1 hour at room temperature, then dehydrated in graded ethanol series and propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections stained with toluidin blue were observed with a light microscope. Ultrathin sections cut in a Porter MT-1 ultramicrotome were stained with 3% uranyl acetate and Reynolds' lead citrate, and examined in a JEOL 12000 EX-type transmission electron microscope.

4) The localization of acid phosphatase

Chick embryos were perfused through the heart ventricle with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution. Anterior portions of the leg bud were taken out and cut into small pieces. They were then immersed in the same fixative for 30 minutes at 4°C and rinsed overnight at 4°C in 0.1 M sodium cacodylate buffer containing 5% sucrose. The tissue pieces were incubated in a similar way to Yamashita et al. (1985) in the modified medium by Gomori (1952) or Novikoff (1963) consisting of 270 mg Na-glycerophosphate, 40 ml of 0.05 M acetate buffer (pH 5.0), 12 ml of 1% Pb(NO₃)₂, 5 g sucrose, and 48 ml of distilled water for 1 hour at 37°C. The tissue specimens were post-fixed in 2% OsO₄ in distilled water, dehydrated in ethanol series and embedded in Epon mixture. Ultrathin sections stained with Reynolds' lead citrate were observed in the electron microscope.

5) Organ culture (Uptake of mouse red blood cells)

An ICR mouse, anesthetized by i.p. injection of pentobarbital sodium, was sacrificed. Blood collected through the left ventricle was diluted two times in Hanks' BSS and fixed with the same volume of 0.1 M Millonig's phosphate buffer solution containing 2.5% glutaraldehyde for 2 hours at 4°C. The sample was centrifuged at 350 G for 10 minutes at 4°C to obtain cell pellets. The cell pellets were resuspended in Hanks' BSS at 4°C and were centrifuged again. This procedure was repeated 10 times in order to remove the glutaraldehyde in the solution. The cell pellets were given as ligands in the following experiment.

Leg buds removed from chick embryos at stage 24 were kept in cold Hanks' BSS. The pellets of mouse red blood cells appropriately diluted in Hanks' BSS were injected in the mesenchymal tissue of the leg buds with a 27 G needle. After the leg buds became red because of the injected mouse red blood cells, the leg buds were excised into small pieces with razor blades. The tissue pieces, which were put in Eagle's minimum essential medium containing 10% fetal calf serum and antibiotics (penicillin, streptomycin and amphotericin B) were incubated at 37°C in 5% CO₂ and 95% air in a water-saturated atmosphere. Three days after incubation, the tissue specimens were fixed with 2.5% glutaraldehyde solution and processed for electron microscopy in the manner mentioned above.

Results

Nile blue stain (Fig. 1a)

Numerous dots were detected in the anterior
area of the leg bud at stages 22, 23, 24 and 25. Density was highest at stage 23. The epidermal region along the apical ectodermal ridge was also stained.

Light microscopy (Fig. 1b, 1c)
Sections of the anterior portion revealed epidermal layer composed of the basal cell and the periderm, and mesenchyme. In the mesenchyme, dermis and subcutis were not clearly identified at any stage examined. Numerous densely-stained particles, 2–3 \( \mu \)m in diameter, were seen among mesenchymal cells and scattered in the intercellular area just beneath the epidermis to an area 50 \( \mu \)m deep. Densely-stained particles were also observed in some cells. There were two kinds of cells which seem to store such densely-stained particles in the cytoplasm. Some, slender in shape, held only one or two of them. The other type of cells, 15 \( \mu \)m or more in size, contained a large amount of densely-stained particles.

Electron microscopy
Epithelial cells
At stage 23 the epithelial layer was double layered with the basal cell and peridermal cell, as similarly demonstrated in the mouse limb bud at day 11.5 of gestation (Nakamura and Yasuda, 1979). Basal lamina was seen beneath the basal cells. No disruption of basal lamina was observed at any stage between 22 and 29.

Mesenchymal cells (Fig. 1d)
At stages 22–29, mesenchymal cells were fusiform in shape extending long cytoplasmic processes. Nuclei were oval and had one or two nucleoli. The cytoplasm was filled with numerous free ribosomes, some elements of Golgi apparatus, mitochondria and a few profiles of rough endoplasmic reticulum. Mesenchymal cells were different from fibroblasts in adult dermis in that rough endoplasmic reticulum was poorly developed and numerous free ribosomes were observed instead. Lysosomes were small in number. A central cilium was often seen. Mitotic figures of mesenchymal cells were common.

A small number of collagen fibers were scattered in the mesenchymal area. Density of collagen fibers was very low in this area compared with that in adult dermis or subcutis.

Necrotic cells (Figs. 1e and 2a–d)
Besides healthy-looking mesenchymal cells, some cells whose electron density was increased were observed in the anterior portion of the leg bud (Figs. 1e, 2a–d) at stages 22–29. They often had numerous electron lucent vacuoles and increased electron dense cytoplasm. They did not form aggre-

gates but were scattered among other mesenchymal cells. These cells were considered to be necrotic. Around such necrotic cells profiles of cell processes of other cells were often recognized.

The electron lucent vacuoles had unit membranes without attachment of ribosomes. The content of the electron lucent vacuoles was not lipid in nature. Dilatation of rough endoplasmic reticulum or Golgi apparatus was not seen. No transitional form was identified between the electron lucent vacuoles and other cytoplasmic organelles such as rough endoplasmic reticulum, smooth endoplasmic reticulum, or Golgi apparatus. Coated vesicles whose diameter was 100 nm were also seen. Rupture of mitochondria was not observed.

In some nuclei of dying cells, chromatin condensation was observed. Heterochromatin, euchromatin and nucleoli could not be identified, whereas outer and inner nuclear membrane were preserved (Fig. 2a). Homogeneously stained round structure, 1–2 \( \mu \)m in diameter, and dot-like granular substances filled some parts of the nucleus. The dot-like substances were sometimes observed to be aligned in a parallel way (Fig. 2b).

In some necrotic cells plasma membrane infolded into the cytoplasm, and some parts of the cytoplasm were cleaved into several fragmented pieces. Some parts of the plasma membrane were ruptured (Fig. 2a).

No discontinuity or rupture of basal lamina was seen even in the area where necrotic cells were close to the basal lamina.

Some necrotic cells, which had dense cytoplasm and electron lucent vacuoles, were observed to have vacuoles containing pieces of necrotic cells (Fig. 2d).

Cells storing cell debris (Figs. 1e, 3a and 3b)
Under light microscope, two kinds of cells were observed to store densely-stained substances in the cytoplasm. Concerning the cells which stored one or two of them, electron microscopy revealed that they stored a small number of membrane-bounded vacuoles, 1–3 \( \mu \)m in size, containing cell debris (cells which are marked by large letters, M in Figs. 1e, 3a) and that this kind of cell had an attenuated cell body, numerous free ribosomes, poorly developed endoplasmic reticulum, and some elements of Golgi apparatus. The cells did not have pseudopods.

The other type of cells, was the large cells with many dense particles at the light microscopic level. It was revealed that they had a large number of membrane-bounded vacuoles containing necrotic cells (Fig. 3a). The large cells, 10–15 \( \mu \)m or more in size and polymorphic in shape, extended pseudopods and microvillus-like cytoplasmic processes. The pseudopods were observed to surround necrotic
cells and often closely apposed to the plasma membrane of necrotic cells which were lying free in the mesenchyme (Fig. 3b).

The contents of some vacuoles were easily identified to be some parts of necrotic cells, which looked similar compared with necrotic cells floating in the mesenchymal area. The contents of other vacuoles seemed to be degraded to some extent. Most vacuoles were 2–3 μm in size, although some vacuoles exceeded 5 μm in diameter. Some large vacuoles were electron lucent with no debris of necrotic cells.

In some of these cells, the cytoplasm was so fully occupied with vacuoles containing cell debris of necrotic cells that profiles of the nuclei were often distorted (Fig. 3a). In the cytoplasm other than the vacuoles, there were some elements of rough endoplasmic reticulum, well-developed Golgi apparatus, mitochondria with rich cristae, coated pits and vesicles. Large and small lysosomes were also recognized in the cytoplasm. In addition to these membrane-bounded organelles, bundles of fine filaments were also seen in the cytoplasm.

Some necrotic cells which were taken up in vacuoles of large cells were observed also to have vacuoles containing necrotic cell debris (Fig. 3b).

No inflammatory cells such as granulocytes or lymphocytes were observed to be accumulated in the connective tissue spaces of the anterior necrotic zone at any stage examined.

**The localization of acid phosphatase (Figs. 4a, b)**

In healthy-looking mesenchymal cells, some cisternae of Golgi apparatus and some cytoplasmic granules were positively stained for acid phosphatase. Necrotic cells were never stained diffusely in the cytoplasm or in the nucleus but only in some cytoplasmic granules (Fig. 4a). The electron lucent vacuoles which appeared during necrotic process were negatively stained.

In cells storing cell debris in one or two vacuoles, reaction products for acid phosphatase were localized in the vacuoles, in some cisternae of Golgi apparatus and in some cytoplasmic granules.

In large cells filled with vacuoles containing cell debris of necrotic cells, acid phosphatase was positive in some of the vacuoles as well as in some cisternae of Golgi apparatus and in some cytoplasmic granules (Fig. 4b).

**The fate of large cells containing necrotic cell debris in their vacuoles (Figs. 5a, b)**

In order to see the fate of large cells filled with vacuoles containing necrotic cell debris, the anterior portion of leg buds at stage 29 was examined (Fig. 5a, b). Population density of mesenchymal cells beneath the epidermis was increased. Numerous large cells, 10–15 μm in diameter, filled with a large number of vacuoles were still observed as well as necrotic cells lying free in the interstitial area. They also had microvillus-like cytoplasmic processes, pseudopods, well-developed Golgi apparatus, some elements of rough endoplasmic reticulum and lysosomes. The size of the vacuoles was 1–10 μm. The contents of the vacuoles were not similar to those at stage 23, when vacuoles as large as 10 μm in diameter were quite rare. In some vacuoles, only some elements of membranes or some organelles remained, and fractions of unit membrane formed whorl-like structures (Fig. 5a). Some vacuoles in the cytoplasm were empty. Cells smaller than 10 μm in size were also observed to store numerous membrane-bounded vacuoles, 2 μm or less in size (Fig. 5b). The vacuoles had electron dense cores and flocculent substances around them. The smaller cells were characterized by their pseudopods, microvillus-like cytoplasmic processes, lysosomes, well-developed rough endoplasmic reticulum, well-developed Golgi apparatus, mitochondria, microfilament bundles, and coated pits or vesicles. They had many features in common with the large cells mentioned above, except for the size of the cell, or size of vacuoles. This kind of smaller cell was not observed at stage 23.

In addition to large cells containing vacuoles, cells with one or two vacuoles containing cell debris were observed. Necrotic cells floating in the interstitial space were still seen, though their number decreased.

**Uptake of mouse red blood cells in organ culture (Figs. 6a, b)**

It was observed in the organ culture experiment that red blood cells injected in the limb bud were taken up into membrane-bounded vacuoles of some cells in the mesenchyme (Fig. 6a), whose rough endoplasmic reticulum and Golgi apparatus were moderately developed. Most red blood cells taken up in the vacuoles were 5–7 μm in size. Formation of large cells containing numerous vacuoles filled with red blood cells was quite rare, but it was observed that in some cells several red blood cells were taken up in vacuoles (Fig. 6b).

**Discussion**

This is the first report to deal with the changes occurring in the anterior necrotic zone of leg bud in the chick embryo at the ultrastructural level. Dying cells in the anterior necrotic zone were characterized by numerous electron lucent vacuoles in the cytoplasm as well as by increased electron density of the cytoplasm and the nucleus. These changes were also described by Hurle and Hinchliffe.
cytes in the bone marrow (Van Furth 1970; Van Furth in peripheral tissues. Tajima et al. (1990) also con-

cluded, it is considered that macrophages appeared in the adult animal (Yamashita et al. 1985). Macrophages in the fetus (Takahashi et al. 1983) which support the idea of local proliferation of macrophages. This notion is widely accepted, though there are some reports concerning the origin of macrophages has been made.

During the dying process, acid phosphatase was never distributed evenly in the cells. The reaction products were confined only to some granules and some elements of Golgi apparatus, and were never found in electron lucent vacuoles. The history of suicide bag hypothesis is described in the review of developmental cell death by Clarke (1990), but it seems that the hypothesis cannot be applied to programmed cell death in the anterior necrotic zone as judged by the localization of acid phosphatase.

The electron lucent vacuoles were membrane-bounded. It is suggested that the vacuoles derive from membranous organelles, such as rough and smooth endoplasmic reticulum, Golgi apparatus, and small vesicles. However, dying cells whose cytoplasm has numerous electron lucent vacuoles still possessed the membranous organelles mentioned above. And no transitional form between electron lucent vacuoles and other membranous cytoplasmic organelles was seen in dying cells. In this study it is difficult to identify the origin of the electron lucent vacuoles in dying cells.

Most mesenchymal cells were more than 5 μm in size. However, neither the profiles of necrotic cells in the mesenchyme nor the vacuoles containing necrotic cells rarely exceeded 5 μm. It is suggested that fragmentation or shrinkage of the necrotic cells takes place during the dying process. In fact, furrows and invaginations observed in the cytoplasm of some necrotic cells (Figs. 2c, d) imply that the necrotic cells were being fragmented. Even in such occasions, nuclei were not observed to be divided into two portions, as reported similarly by Hurle and Hinchcliffe (1978).

Van Furth proposed the mononuclear phagocyte system, in which macrophages derive from monocytes in the bone marrow (Van Furth 1970; Van Furth and Cohn 1968; Van Furth et al. 1972). This notion concerning the origin of macrophages has been widely accepted, though there are some reports which support the idea of local proliferation of macrophages in the fetus (Takahashi et al. 1983) and in the adult animal (Yamashita et al. 1985). In the embryos whose hematopoiesis has already started, it is considered that macrophages appeared in peripheral tissues. Tajima et al. (1990) also con-

firmed that in the chick embryo, the immature macrophage first appeared in blood islands at stage 11. In the rat embryo, fetal macrophages begin to appear in the subepidermal mesenchyme after about 12 days of gestation (Takahashi et al. 1983). Judging from ultrastructural features such as the existence of pseudopod-like protrusions, microvillus-like cytoplasmic projections and lysosomes, the large cells with numerous vacuoles containing cell debris may well be called macrophages.

The necrotic cells were surrounded by numerous cell processes of other cells, such as typical mesen-

chymal cells or macrophages. It is recognized that macrophages engulfed necrotic cells by phagocytosis as indicated in Fig. 3b, stored them in cytoplasmic vacuoles (phagosomes) and digested the contents after the fusion of lysosomes with the phagosomes. Acid phosphatase-positive vacuoles in macrophages are considered to be phagolysosomes. In thyroid follicular epithelial cells, it was shown that lysosomes gathered to fuse with vacuoles containing red blood cells or large latex beads which were phagocytosed from the apical side of the epithelial cells (Miyagawa et al. 1983).

No other previous works have reported that macrophages have the ability to take up and store so large a number of necrotic cells in the area of physiological cell death in the limb bud. It was shown that the Kupffer cell, the liver macrophage, in the adult mouse which was given i.v. India ink particles or large and small polystyrene latex beads took up the particles by micropinocytosis or by phagocytosis and stored them in their cytoplasmic vacuoles (Fujita et al. 1983; Yamashita et al. 1985). Kupffer cells containing vacuoles stuffed with carbon particles or latex beads gathered in the sinusoidal space and formed aggregates. In the present study, aggregates consisting of macrophages were not formed.

In the organ culture experiment, it can be con-

sidered that cells were not recruited from the bone marrow, because there was no blood circulation. The experiment has shown that cells which can take up red blood cells do exist in the mesenchymal area. They may be mesenchymal cells, or macrophages which already existed in the connective tissue space as "resident macrophages". The fact that vacuoles containing the red blood cells were 6–7 μm in size may imply that the red blood cells can be taken up by phagocytosis and that fragmentation of cells is not a requisite for a necrotic cell to be taken up.

As seen in Fig. 5a, some vacuoles containing necrotic cells in macrophages at stage 29 seem to be larger than those at stage 23. This may be because some of the vacuoles fused each other to form larger vacuoles. In addition, the fact that only membrane
components remained in the vacuoles may indicate that the contents were degraded to some extent. It is not confirmed whether the membrane components of cell debris can be completely digested or not. The cell in Fig. 5b can be regarded as a macrophage in that it has many ultrastructural features in common with large cells containing numerous vacuoles stuffed with necrotic cells. The fact that this kind of cell had smaller vacuoles with dense cores may imply that the contents of the vacuoles were further degraded and digested.

In addition to macrophages, some mesenchymal cells were observed to have a few vacuoles containing cell debris. Concerning the origin of the vacuoles, Hurle and Hinchiliffe (1978) insisted that they were autophagic in nature and that these autophagic vacuoles reflected the very early stage of cell necrosis. In the review on cell death by Clarke (1990), the observation by Hurle and Hinchiliffe (1978) was classified type 2 (autophagic cell death) because of the existence of abundant autophagic vacuoles.

As to the origin of the vacuoles seen in mesenchymal cells, another possibility can be considered. That is, mesenchymal cells engulfed fragments of other necrotic cells and stored them in the cytoplasmic vacuoles. Phagocytosis is regarded as the ability to take up material whose diameter is more than 1 μm (Fawcett 1986), whereas pinocytotic activity is to endocytose materials whose diameter is less than that. It is difficult to recognize that embryonal mesenchymal cells are almost the same as fibroblasts in adult tissues in the ability of taking up foreign materials. Both fibroblasts in dermis of adult mouse skin (Fujita et al. 1988) and keratinocytes in the cornea of adult rabbit eyes (Fujita et al. 1987) were shown to have the ability of micro-pinocytosis to take up foreign bodies whose diameter is less than 1 μm, such as India ink particles or small polystyrene latex beads, whereas macrophages in dermis have the ability to take up large (2 μm) latex beads by phagocytosis as well as small latex beads and India ink particles. In the present study, mesenchymal cells were not like adult fibroblasts in that they had a smaller number of rough endoplasmic reticulum. As for the mesenchymal cells which had one or two vacuoles containing cell debris, the size of vacuoles were not always less than 1 μm. It is considered that mesenchymal cells in the embryonic stages have the ability of phagocytosis. However, macrophages could be distinguished from mesenchymal cells due to the presence of pseudopods, microvillus-like cytoplasmic processes and lysosomes. Yajima (1988) also reported that fibroblasts in adult human gingiva take up and dispose of collagen fibrils as well as secreting them.

It is an interesting fact that cells which had a vacuole containing cell debris were shown also to be dying with numerous electron lucent vacuoles. In Fig. 2d, the necrotic cell had a vacuole whose content was a cell debris made of electron lucent vacuoles. This fact shows that the cell, whether it is a mesenchymal cell or a macrophage, started the process of cell death after taking up other cell debris. If the cell is a mesenchymal cell, there may not be any clear distinction between dying cells and phagocytosing cells. And if the cell in Fig. 2d is a macrophage, it may be considered that the macrophage also dies, at least, at the anterior necrotic zone.

It is considered that the macrophage plays a major role in the disposal of necrotic cells and that the mesenchymal cell also takes some part in the process. However, the endocytotic activity of the mesenchymal cell has not been well clarified. Further study is necessary on the mesenchymal cell in this respect. Komuro (1990) has insisted that the system of the fibroblast must be re-evaluated and proposed that fibroblast-like cells including fibroblasts can be categorized into subtypes depending on their main functions. The mesenchymal cells, many of which give rise to fibroblasts, must be re-evaluated as well in many aspects of their functions.

References

Physiological Cell Death in Chick Leg Bud


Explanation of Figures

Plate I

Fig. 1a–e. Leg bud at stage 23.

a: Micrograph under a dissection microscope of a leg bud (Nile blue staining, bar = 0.5 mm). Uptake of Nile blue is seen in the anterior part of the leg bud (arrowheads) and along the apical ectodermal ridge (small arrows).

b: Light micrograph of an anterior portion of a leg bud (JB-4 section, hematoxylin and eosin stain, bar = 10 μm). The section was cut coronally. Numerous darkly-stained particles (arrowheads) are seen beneath the epidermis. The epidermis is composed of two cell layers.

c: Light micrograph of an anterior necrotic zone of a leg bud (Semithin section, toluidin blue staining, bar = 5 μm). Densely stained particles are noticed beneath the epidermis. Some of them are seen scattered and lying free in the interstitial tissue (small arrows). Some cells contain one densely stained particle in their cytoplasm (arrowheads). Aggregates of the densely stained particles are seen (A). E, epidermis.

d: Electron micrograph of a typical healthy-looking mesenchymal cell in the anterior portion of a leg bud (bar = 1 μm). The cell is extending some cytoplasmic processes. A close association of the cytoplasmic process is noticed with a neighboring cell process (small arrow), although the nature of the association is not clearly defined in this magnification. Profiles of Golgi apparatus and a coated vesicle are observed.

e: Electron micrograph of an anterior portion of a leg bud (bar = 2 μm). Beneath an epidermal layer, a densely stained necrotic cell (N) floating in the mesenchyme and a cell (M) containing vacuoles filled with cell debris are observed. A few profiles of collagen fibers are also seen.
Plate II

Fig. 2a–d. Electron micrographs of necrotic cells in the anterior necrotic zone of a leg bud at stage 23 (bars in a, b, c, and d = 1 μm).

a: The nucleus and cytoplasm are clearly distinguished with preserved outer and inner nuclear membranes (arrowheads). A homogeneously stained structure (H) and dot-like granules are seen in the nucleus. In the cytoplasm, fine granules which look like free ribosomes are seen. Rough endoplasmic reticulum can be identified. Electron lucent vacuoles which are composed of limiting membranes are seen. Some parts of plasma membrane are ruptured and communicate with the electron lucent vacuoles in the cytoplasm (arrow).

b: Granules aligned in parallel arrays seen in the nucleus (arrowheads). Small arrows indicate nuclear membrane.

c: Furrows of the plasma membrane of a necrotic cell (arrowheads). The necrotic cell seems to be fragmenting. Electron lucent vacuoles, mitochondria, free ribosome-like granules, coated vesicles, fine filament bundles are observed. Cell process in contact with the necrotic cell is also noticed (small arrows).

d: A necrotic cell which has a vacuole (D) filled with cell debris. The cell debris in the vacuole (D) also has profiles of electron lucent vacuoles. Notice the narrow portion of the cytoplasm, which may suggest a process of fragmentation (arrowheads). N, nucleus.
Plate III

Fig. 3a,b. Electron micrographs in the anterior necrotic zone of a leg bud at stage 23 (bars in a and b = 2 μm).

a: Cells filled with a large amount of cell debris in vacuoles. A mesenchymal cell (M) is observed to have a vacuole containing cell debris (arrowhead).

b: Pseudopods protruding from a large cell. They surround and attach necrotic cells, which have electron lucent vacuoles and electron dense cytoplasm. A necrotic cell (N) is completely surrounded by the pseudopod and is observed to have a vacuole (V) filled with cell debris. Cell debris in a vacuole (D) seems to be degraded.
Plate IV

Fig. 4a,b. Electron micrographs showing the localization of acid phosphatase activity in the anterior necrotic zone at stage 24 (bars in a = 1 μm; b = 2 μm).

a: A necrotic cell and a cell surrounding the necrotic cell. The acid phosphatase activity is localized in some cisternae of Golgi apparatus (arrowhead) and some granules in the surrounding cell, and in some granules in the necrotic cell. The numerous electron lucent vacuoles in the necrotic cell are negative for acid phosphatase activity.

b: A large cell which has numerous vacuoles containing necrotic cell debris. Acid phosphatase activity is localized in some of the vacuoles, while other vacuoles are negatively stained.

Fig. 5a,b. Large cells containing numerous vacuoles filled with cell debris in the anterior necrotic zone at stage 29 (bars in a and b = 2 μm).

In a, the content of the vacuole seems to be degraded, and some whorl-like bodies made of membrane components are observed in these vacuoles (arrowheads). Microvillus-like cytoplasmic processes are seen. Numerous lucent vacuoles are noticed as well. Golgi apparatus is well developed. In b, the size of the cell is not so large as the cell in a. The content of vacuoles is small in volume. Some vacuoles have electron dense cores and flocculent substances around them. Pseudopods are also observed (arrow).

Fig. 6a,b. Red blood cells taken up in cells three days after the incubation of pieces of leg buds which were injected with red blood cells (bars in a and b = 1 μm). Each red blood cell is enclosed in a vacuole. Note the limiting membrane of the vacuoles (arrowheads). The plasma membrane of the red blood cells is not clear. In a, one red blood cell is engulfed in the cell. In b, several red blood cells are observed in vacuoles. Lucent vacuoles are seen as well.