out on rat liver lysosomes for experimental convenience—provide in vitro evidence consistent with such a suggestion.

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Cell Death during Early Morphogenesis: Parallels between Insect Limb and Vertebrate Limb Development

Abstract. *The complex jointed leg of the adult fly is derived, in the pupal stage, from a simple lobed sac of cells. The gross morphological changes that result in adult shape are effected by a combination of differential cell growth and the programmed death of a large number of imaginal disc cells. Events are closely similar to those occurring in digit formation and limb contouring during vertebrate morphogenesis. In both cases phagocytic blood cells are intimately involved.*

During the development of the vertebrate limb, digit formation is accompanied by programmed cell death, and areas of necrosing cells appear at specific sites and times (*1*). The death and resorption of these cells contribute to the morphogenetic movements which model not only the digits (Fig. 1A) but also the contours of the thigh and upper arm. In the duck the distribution of such areas of necrosis is correlated with the degree of webbing (*2, 3*). Between digits one and two, which in the fully formed foot are not connected by webbing, the interdigital zone of the footplate has V-shaped wedges of necrosis extending from the distal margin and involving some ectodermal cells and a high percentage of mesenchymal cells. In contrast, between digits two and three, and three and four, which are webbed in the adult, only shallow zones of necrosis occur. Only the distal regions of the interdigital tissue degenerate, the rest remaining as web. Inhibition of necrosis by injection of Janus green results in high frequency of syndactylism and in an absence of interdigital macrophages (*4*).

In limb development of *Sarcophaga bullata*, the flesh fly, a remarkably similar sequence of events occurs (Fig. 1B). The adult fly leg is composed of coxa, trochanter, femur, tibia, tarsus (composed of five tarsal segments), and a terminal pretarsus; the latter is composed of two claws and two footpads, or pulvilli, which the fly uses to adhere to the substratum. The tarsal segments are separated by joints that are deeply indented, and the two pulvilli of the pretarsus are also separated as far as their bases. Yet these structures are derived, at the beginning of adult development, from a simple sac of epidermal cells which follow the outlines of, and are closely apposed to, the pupal cuticle which they have themselves recently secreted. This simple sac of cells, together with the adhering pupal cuticle, is slightly lobed at the level of the future tarsal joints, and also very slightly lobed distally (*5*).

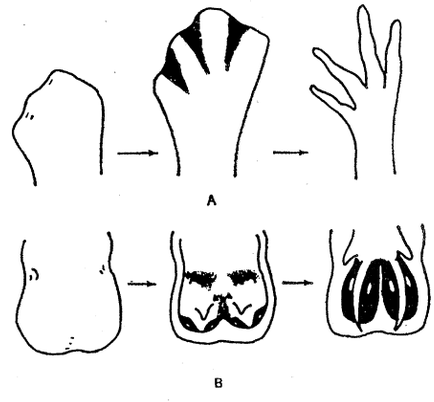

Fig. 1. Areas of cell necrosis in vertebrate and insect limbs during morphogenesis. (A) Diagram of relation between cell necrosis and digit formation in the chick (*3*). (B) Diagram of relation between cell necrosis and pulvillar formation in the fly. Areas of cell death denoted by the lightly shaded area; rapidly growing giant dorsal cells, black; unshaded nuclei, white.

The leg is present in the larval fly as an imaginal disc. At pupation, the pupal cuticle is secreted by the epidermal cells, and phagocytic hemocytes, gorged with larval tissue fragments, are forced into the leg as it is extended with hemolymph. Tracheoles, also epidermal, are present, and nerves extend as far as, but not into, the pretarsus. Since the tarsal segments are devoid of muscles in the adult, no mesodermal elements exist there other than the hemocytes.

Within 24 hours after pupation of the larval fly, the leg epidermis is withdrawn from the pupal cuticle, and extensive cell division is seen. By this time, certain cells can be distinguished by their larger nuclei and polytene chromosomes; these include five cells that migrate in file to form the distal region of each claw, two cells that will form basal plates, and four cells that will reach giant proportions and will each be responsible for the secretion of half the cuticle of the dorsal surface of each footpad (*6*). On the tarsal segments, future trichogen (hair) and tormogen (socket) cell nuclei are evident, also showing a low degree of polyteny. For those cells that will become large and have polytene chromosomes, increase in cell size and DNA replication continues from day 2 through into day 4 (*7*). Extensive cell division occurs ventrally in the pretarsus during day 2 and into day 3. The products of these cell divisions differentiate, during day 3, into tenent hair cells, responsible for forming the cuticular hairs of the ventral adhesive surface of each of the two pulvilli.

During days 3 and 4 there is virtually no cell division in the leg. This marks the period of cell growth, cell differentiation, and cell movement, bringing about the gross morphological changes that transform the simple lobed structures of a late day 2 pupa into the well-contoured adult form that is established by late day 4. It now appears that this same period is marked by dramatic cell death. Tenent cells break down later at day 9 to 10, and giant footpad cells break down at the time of their emergence. This is not surprising for

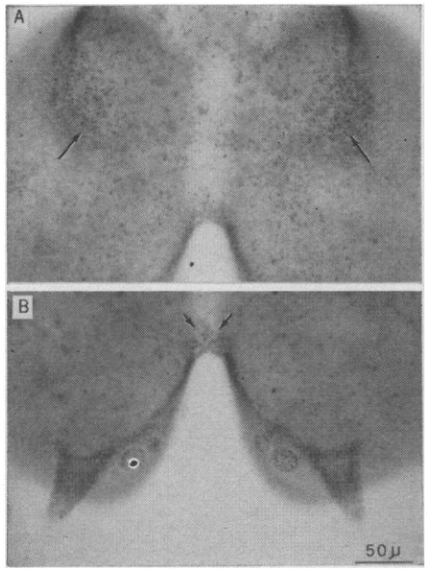

Fig. 2. Pretarsus of *Sarcophaga bullata* showing areas of cell necrosis. (A) Proximal region of pretarsus in dorsal view, Feulgen stained at day 3, the beginning of claw and pulvillus formation; the pycnotic nuclei of the dying cells are seen in two areas laterodorsally. (B) Distal region of a pretarsus of same age, showing third area of necrosis, at point of continuing retraction, in the midline. This region is equivalent to the wedges of necrosing cells in the vertebrate interdigital areas. Claw formation is only beginning. By the time these morphogenetic processes are completed, indentation will extend to the base of the pretarsus, the claws will extend from the base of the pretarsus to beyond the pulvilli distally, and most of the cells that lie dorsally in (A) and (B), between the base of the pretarsus and the base of the developing claws, will have died and been ingested by phagocytic hemocytes.

their secretion of adult cuticle is, by this time, over. Cell death during early morphogenesis is, in contrast, wholly unexpected for the insect, although well known for the vertebrates.

Apparently, during the course of days 3 and 4, almost the entire cell population of the dorsal surface of the pretarsus of a new day 1 pupa (except for those cells contributing to the claws, basal plates, and the four future giant dorsal cells) will die, and their contents will be engulfed by the phagocytic hemocytes that will later be involved in the ingestion of tenent and giant dorsal cells (*8*). In contrast to the high mortality among the dorsal cells of the pretarsus, not one pycnotic cell has been observed in the ventral region. The death of these dorsal cells does not occur synchronously; over 48 hours, cell death and resorption occur progressively as the giant cells of each future footpad grow and extend proximally over the dorsal footpad surface. The dying cells are contained in two areas of necrosis situated dorsolaterally at the base of the pretarsus (Fig. 2A). At these areas cells die, sink into the hemocoele, and are engulfed by the blood cells, thus allowing room for further continuing extension of the giant cells. The last cells to enter the area of necrosis will be those immediately proximal to the giant cell cytoplasm.

Another area of necrosis lies in the midline of the distal extremity of the pretarsus, being similar to the interdigital wedges of the chick limb. Progressive cell death and resorption results in deeper and deeper invagination (Fig. 2B), eventually producing the two pulvilli, which can be compared to two vertebrate digits. The necrosing cells stain with the same basic dyes as do those of chick and duck, and the nuclei are seen to be pycnotic with Feulgen stain. The phagocytic hemocytes are clearly seen with their enclosed debris, being remarkably similar to the vertebrate macrophages in appearance. Cell death is also involved in the contouring of the tarsal segments, the deeply indented joints of the adult being formed from the barely lobed pupal precursor.

In the vertebrate limb the death clocks function on time even when the tissue is transferred to a host of different age. Since this timing of the vertebrate death clock is independent of external factors, it has been specifically contrasted (*3*) with events that occur in the insect during metamorphosis. At pupation in the insect, a "schedule of hormonal conditions, innocuous to other cells" (*3*) evokes the death response from certain cells that have differentiated the competance to respond. In this category belong all those tissues whose cells have undergone cell growth rather than cell division during larval life, including salivary glands, muscles, midgut epithelium, and large expanses of epidermis. Imaginal disc cells have long been considered as typical examples of cells that divide during larval life and do not die at pupation. This generalization now appears incorrect; large numbers of imaginal disc cells do in fact die during early adult morphogenesis. However, they are not programmed to do so at the time that other larval cells break down at pupation. Instead, their progressive death occurs during the 2nd, 3rd, and 4th days after pupation.

In many respects the footpad cells behave like moth imaginal muscle cells which break down after emergence. These muscle cells receive the hormonal death signal at pupation, but respond very much later, after adult emergence, when nerve impulses to them suddenly cease (*9*). However, the present pretarsal cells are not individually innervated. Since their behavior is similar to that of the vertebrate limb cells, one cannot ignore the possibility of their responding to some factor or factors comparable with the "trans filter" factors found to be emitted from the leg and wing mesoderm of the chick. After prolonged exposure to these "trans filter" factors in vitro, the death clock can be turned off and the chick limb cells can be induced to lead an indefinitely prolonged life (*10*).

It seems likely that events shown for the fly occur also in other insects in the development of such structures as highly contoured legs, antennae, and mouthparts. Not only are the blood cells remarkably similar to the vertebrate macrophages, but death clocks and their genetic programming seem to function in establishing shape in the insect as they do in vertebrate morphogenesis.

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