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Amebocyte Production Begins at Stage 18 During Embryogenesis in *Limulus polyphemus*, the American Horseshoe Crab

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Abstract. *Limulus polyphemus,* the American horseshoe crab, has a single type of circulating blood cell, the granular amebocyte, which is the horseshoe crab's primary cellular defense against microbial infection. On exposure to gram-negative bacteria or their endotoxins, the amebocytes degranulate, releasing the clotting protein coagulogen and a number of proteases. The protease cascade converts the soluble coagulogen to insoluble coagulin, which forms fibrous clots that seal off the site of infection. The first description of this clotting reaction in the 1950s initiated development of *Limulus* amebocyte lysate and spurred an intensive study of the amebocytes. However, the site or sites and timing of amebocyte production have yet to be determined.

We report here that during embryonic development in *Limulus polyphemus,* amebocyte production begins at stage 18. The first amebocytes detected are found in developing hemocoel cavities, and the cells may derive from previously undifferentiated yolk nuclei.

Introduction

Granular amebocytes, the sole circulating blood cells in the hemolymph of *Limulus polyphemus,* have been studied for nearly 50 years. The amebocytes are the horseshoe crab's primary line of cellular defense against infection by the many gram-negative bacteria that share its marine habitat. Exposure to gram-negative bacteria or their endotoxins causes the amebocytes to degranulate through an exocytotic pathway (Bang, 1956; Dumont *et al.,* 1966; Armstrong and Rickles, 1982; Levin, 1985b; Ornberg, 1985). The granules contain a number of proteases and the primary clotting protein coagulogen. The proteases initiate a cascade that results in the conversion of the soluble coagulogen to insoluble coagulin. The coagulogen assembles into fibrous clots that seal off the site of infection, trapping the invading microorganisms (see Iwanaga, 2002, for review). Frederick Bang first described the clotting reaction in response to gram-negative bacteria in the 1950s, and subsequent studies by Bang, Levin, and colleagues (see Levin, 1985a, for review) led to the development of *Limulus* amebocyte lysate (LAL). LAL is widely used to test for the presence of endotoxins in intravenous fluids and drugs, vaccines, and solutions used in the decontamination of medical instruments. The molecular details of the protease cascade and the clotting reaction have been determined, and many of the genes involved have been cloned (Iwanaga, 2002).

Despite extensive study, the site of hemopoiesis has yet to be identified. Hilly and Gibson (1989) reported production of amebocytes in cultures of excised gill tissue; however, this has not been confirmed. Analysis of mRNA expression patterns in heart, muscle, coxal gland, brain, hepatopancreas, and midgut has ruled out these tissues as likely sites for amebocyte production (Miura *et al.,* 1995; Agarwala *et al.,* 1996). The consensus among those who study horseshoe crab blood is that the amebocytes are probably produced within the connective tissues, as has been suggested for other invertebrates that lack specific hematopoietic organs (Sawada and Tomonaga, 1996); however, this has yet to be demonstrated experimentally.

This paper addresses another major unanswered question about amebocyte production: when does it begin? Circulat-

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ing blood cells can be seen in late-stage embryos and newly hatched first instar (trilobite) larvae (Bang, 1979). Liang *et al.* (1990) reported that blood cell production in *Tachypleus tridentatus* Leach (1819), the Japanese horseshoe crab, begins "at the stages of segmentation and appearance of limb buds." This appears to correspond to stages 13–15 in the staging scheme described by Sekiguchi (1973). Sekiguchi divided horseshoe crab embryogenesis into 21 stages, based on external morphological changes. Comparable morphological events occur in *T. tridentatus* and *L. polyphemus* embryos (Sekiguchi *et al.*, 1982, 1988). Assuming that similarity in external appearance also correlates with comparable internal development, we predicted that amebocyte production in *L. polyphemus* would also begin by embryonic stage 15. However, we show here that blood cell production in *L. polyphemus* does not begin until embryonic stage 18.

Materials and Methods

Embryo collection and processing for histological analysis

Fertilized horseshoe crab eggs were collected from beaches around Tampa Bay, Florida. The breeding season in Tampa Bay begins in late March and extends through mid-October. The eggs were collected by marking the location of mating pairs on the beach at high tide and then returning to the site 3 to 4 h later. The eggs were typically located 10–12 cm beneath the surface. In the laboratory, the eggs were separated from the bulk of the sand and maintained in plastic tubs in filtered seawater at 30 °C. Eggs were selected at timed intervals and stained with 0.02% neutral red to determine the stage of development as described by Sekiguchi *et al.* (1982).

Embryos (stages 12–21) and trilobite larvae were fixed overnight at 4 °C in a modified Bouin's fluid or in 3% formaldehyde in seawater, then dehydrated, infiltrated, and embedded in Unicryl as described previously (Kimble *et al.*, 2002). Sections of 4 to 5 μm were cut, using glass knives, and mounted on heated glass slides. Sections were stained with Giemsa, iron hematoxylin, or with an anti-coagulogen antibody, to identify amebocytes.

Antibody production

Polyclonal antibodies against coagulogen were raised in New Zealand White rabbits. Coagulogen was purified from amebocytes following the protocol of Miyata *et al.* (1984), as modified by P.B. Armstrong (University of California, Davis) and colleagues (pers. comm. to Y.C.). Blood samples were obtained from *L. polyphemus* adults. The amebocytes were allowed to settle overnight, and the supernatant (serum) was decanted. The cells were resuspended in 10% acetic acid and frozen. After thawing, the cell slurry was sonicated to release the coagulogen granules, and the granules were collected by centrifugation. The granule pellet was resuspended in ammonium bicarbonate and the pH adjusted to 7.0. Proteins were precipitated with 40% ammonium sulfate, resuspended in ammonium bicarbonate, dialyzed, and then run over a Sephadex G100 column. Fractions were collected and total protein concentration was checked by the BCA (bicinchoninic acid) assay. Fractions were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Column-purified proteins were mixed with adjuvant (complete for the first injection and incomplete for subsequent (boost) injections) and injected subcutaneously into rabbits. Boost injections were given at intervals of about 2 weeks. Following the third and subsequent boosts, blood samples were taken, and the serum was tested by protein blot analysis against column fraction 24 (fraction used for immunization), and against whole blood proteins from horseshoe crabs purchased from the Marine Biological Laboratory (Woods Hole, MA), or collected in Tampa Bay, for the presence of anti-coagulogen antibodies. Following the fourth boost (fifth injection), the rabbits were bled weekly for 4 months, after which they were euthanized and bled out. The serum was collected, aliquoted, and frozen at −80 °C.

Immunolocalization and microscopy

Sections were blocked using 5% normal goat serum in TBS (Tris buffered saline) for 1 h, and incubated with antiserum (1:10,000 final dilution), preimmune serum (1:5,000), or 5% goat serum, in TBS overnight at 30°C. Bound primary antibodies were detected by sequential incubation of the sections in a biotinylated anti-rabbit secondary antibody (1:30), ExtrAvidin alkaline phosphatase (1:30), and *Fast*® fast red TR/napthol AS-MX (Sigma). Cleavage of *Fast* fast red by alkaline phosphatase produces a bright red precipitate. Antibodies were diluted in TBS-tween, and the ExtrAvidin alkaline phosphatase was diluted in TBS. The *Fast* fast red TR/Napthol AS-MX was prepared according to manufacturer's instructions. Washes following the primary and secondary antibodies were in TBS-tween. Washes following the binding of the alkaline phosphatase were in TBS, with a final rinse in deionized water prior to addition of the *Fast* fast red. The color reaction was stopped after 5 min by rinsing with deionized water. Sections were post-stained with 0.5% methyl green. Coverslips were mounted with glycerol immediately before viewing. Sections were viewed and photographed on a Nikon inverted microscope.

Results

Blood cell production during embryogenesis

Blood cells in staged horseshoe crab embryos were initially identified using the histological stains Giemsa and

iron hematoxylin. Giemsa staining provides easy identification of different tissues, but the amebocyte granules stain only sporadically. Iron hematoxylin reproducibly stains the amebocyte granules, but the staining is similar in appearance to staining of the yolk. Despite these limitations, both staining techniques yielded the same result. Amebocytes were identified in stage 18 and older embryos (Fig. 1A–E), but not in earlier stage animals (not shown).

To confirm that the cells identified as amebocytes in the Giemsa and hematoxylin stained specimens are in fact amebocytes, embryos at the same stages of development were stained using an anti-coagulogen antiserum. On protein blots, the antiserum gives a strong, specific reaction to coagulogen when diluted 1:50,000 (arrowhead in Fig. 2B). In immunohistochemical studies, the antiserum specifically stains the coagulogen granules in amebocytes of trilobite larvae (Fig. 3A, B). In embryos, the antiserum specifically stains cytoplasmic granules in amebocytes beginning at stage 18 (Fig. 3C–E) of embryogenesis. No antibody staining was detected in earlier stage embryos (not shown).

In embryos at stages 18 and 19, the amebocytes were typically located in areas devoid of yolk and other ooplasmic components. These ooplasm-free regions appear to be precursors of the hemocoel cavities. They are typically located dorsal to the ventral plate of the embryo, between the epidermal cell layer and the layer of squamous mesodermal cells that overlays the central yolk mass (Fig. 1A, B). In some sections there also appears to be a second mesoderm layer directly beneath the epidermal cell layer. Thus the cavities may in fact be forming between two layers of mesodermal cells. In addition to the amebocytes, elements of connective tissue are frequently seen within these cavities (Fig. 1A). In embryos at later stages (late stage 20 and stage 21) and in trilobite larvae, most blood cells are found in the developing hemocoel cavities, associated with connective tissue, or within the heart. However, we have

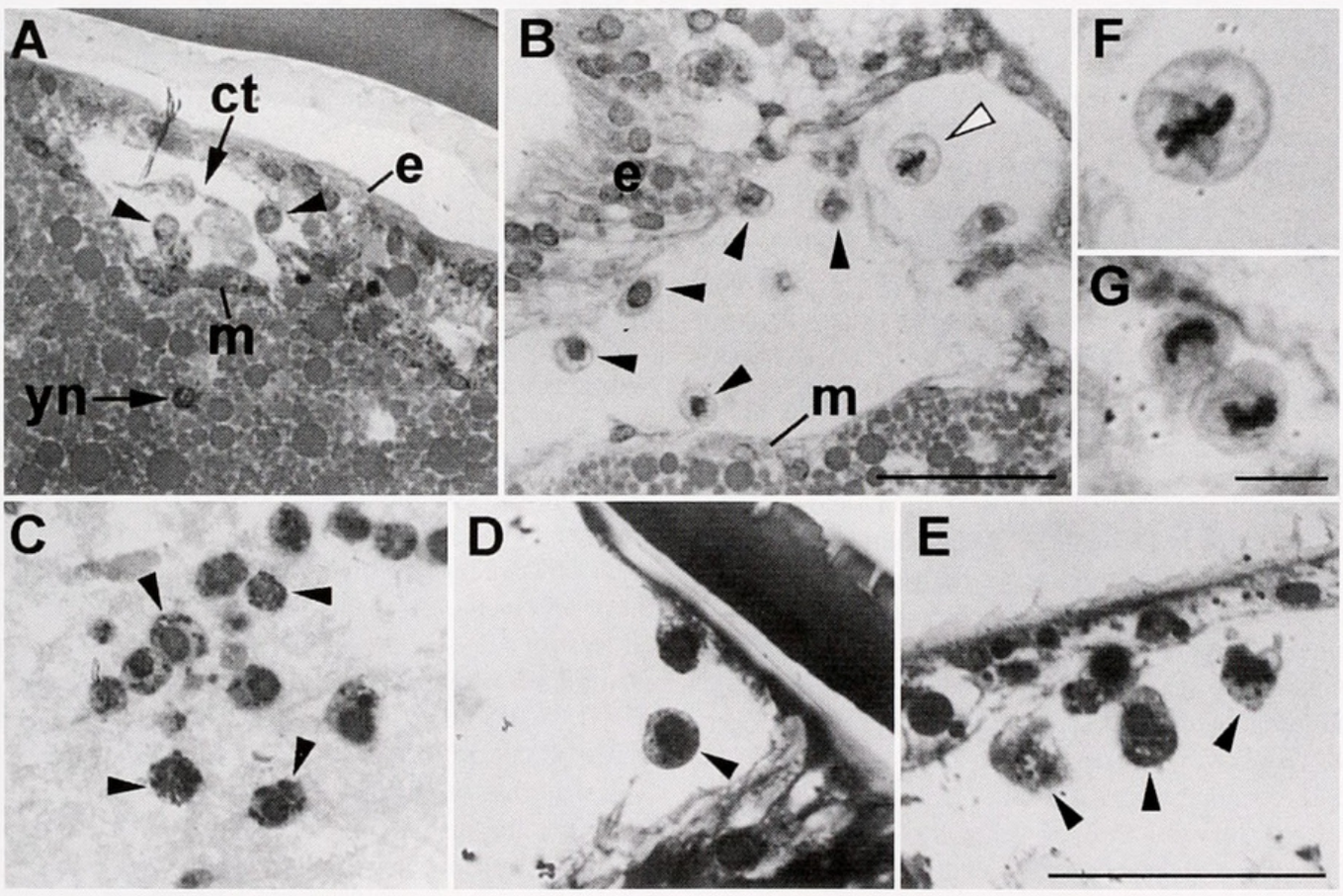

Figure 1. Amebocytes in embryos and larvae stained with Giemsa (A, B) or iron hematoxylin (C–E). (A, B) Sections from stage 18 embryos showing putative amebocytes (arrowheads) in developing hemocoel cavities. Open arrowhead in B indicates a cell that is in metaphase. (C) Section through the heart of a trilobite larva showing the granulated amebocytes (arrowheads). (D) Section from a stage 18 embryo showing two cells in a developing cavity. One has several darkly stained cytoplasmic granules (arrowhead). (E) Section from a stage 19 embryo showing several cells that contain cytoplasmic granules (arrowheads). (F) Higher magnification of the metaphase cell in panel B. (G) Sister cells in telophase from a different section of the same embryo as in B and F. ct, connective tissue; e, epidermal cell layer; m, mesodermal layer; yn, yolk nucleus. A and B are at the same magnification, bar in B = 50 μm. C–E are at the same magnification, bar in E = 50 μm. F and G are at the same magnification, bar in G = 10 μm.

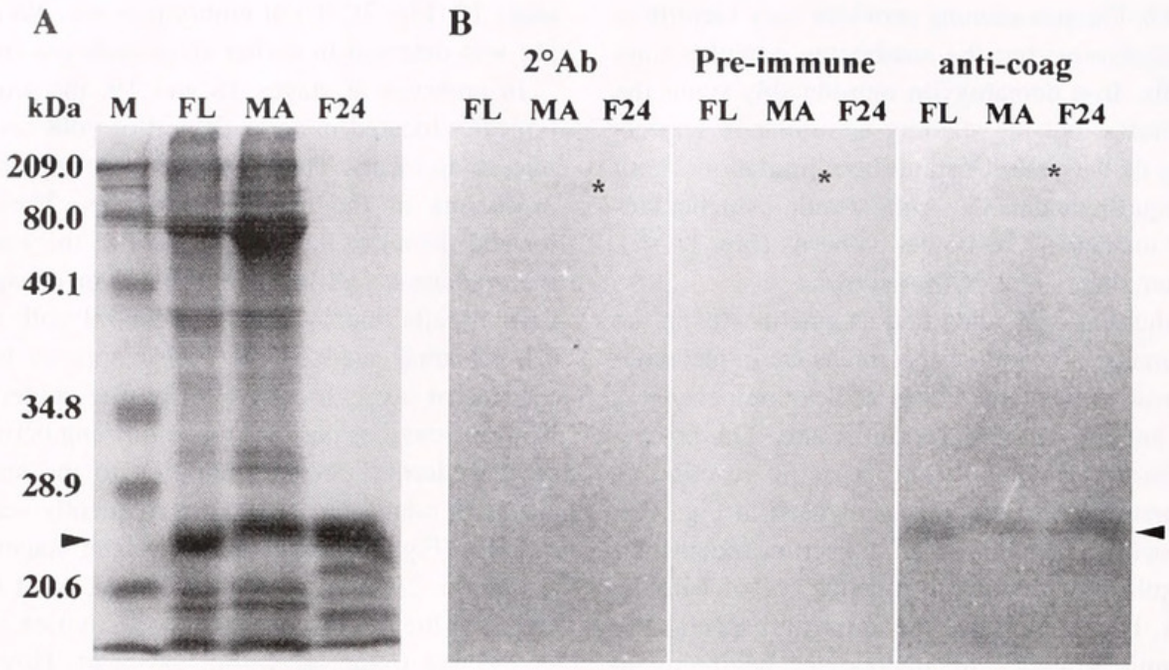

Figure 2. Protein gel and western blot analysis of total blood proteins from horseshoe crabs collected in Cape Cod (MA) and Tampa Bay (FL), and column fraction 24 (F24). (A) Coomassie-stained gel showing the three protein samples and molecular weight markers (kDa). The arrowhead to the left indicates the coagulogen protein band. (B) Blots from protein gels run in parallel with the gel shown in panel A. The blots were probed with the anti-coagulogen serum (anti-coag), pre-immune serum, or with secondary antibody (2°Ab) alone. The arrowhead at the right indicates the position of coagulogen. The asterisk indicates a high molecular mass band in the MA sample that binds the secondary antibody.

also observed blood cells in close association with isolated yolk masses (Fig. 3F, G).

Discussion

Amebocyte production begins at a later stage in Limulus polyphemus *than in* Tachypleus tridentatus

Using a combination of histological stains and an antibody that specifically recognizes the coagulogen protein in amebocytes and on protein blots, we have shown that amebocyte production in *L. polyphemus* begins at stage 18 of development. Amebocytes were identified in all subsequent embryonic stages (stages 19–21, days 7–14 post-fertilization) and in the trilobite larvae, but not in embryos prior to stage 18.

Stage 18 occurs during the 6th day post-fertilization and coincides with the first embryonic molt. At this stage the dorsal half of the embryo is still rounded and lacks distinguishing marks. The ventral side of the embryo is flattened, the prosomal appendages (chelicerae, pedipalps, and walking legs) have begun to lengthen, the stomodaeum is located slightly anterior to the chelicerae, and the lateral organs have begun to develop (Sekiguchi *et al.*, 1982). As mentioned previously, Liang *et al.* (1990) reported the identification of pro-amebocytes in transmission electron micrographs of stage 13–15 *T. tridentatus* embryos. The pro-amebocytes identified by these authors were first seen within the germ band (ventral plate), but in later stages were distributed diffusely in the connective tissue. Liang *et al.* (1990) suggested that the pro-amebocytes derived from the mesenchymal cell layer of the ventral plate. Despite careful examination of our sectioned embryos, we have not identified cells within the ventral plate that appear comparable to the proamebocytes described by Liang *et al.* (1990). However, the amebocytes that we observe in the hemocoel cavities of stage 18 embryos do correspond in appearance to their description of immature amebocytes. That is, the cells have relatively few coagulogen granules, and the granules, at least in some cells, appear to be smaller than granules in mature amebocytes (compare panel 3D with panels 3A and E–G). Additional studies will be necessary to determine if the apparent difference in granule size does in fact indicate cell maturity, or whether it reflects cell-to-cell variation. In addition, the developing hemocoel cavities, in which the immature amebocytes are observed, are typically located near, although not within, the ventral plate. For example, the cavity shown in Figure 1A was located between the ventral plate and the lateral organ. Thus the discrepancy in the location of the earliest amebocytes between the two species may be due to differences in how each group defines the ventral plate, rather than a significant difference in the actual location of the cells.

In agreement with Liang *et al.* (1990), we were not able to identify any obvious hematopoietic tissues in any of the

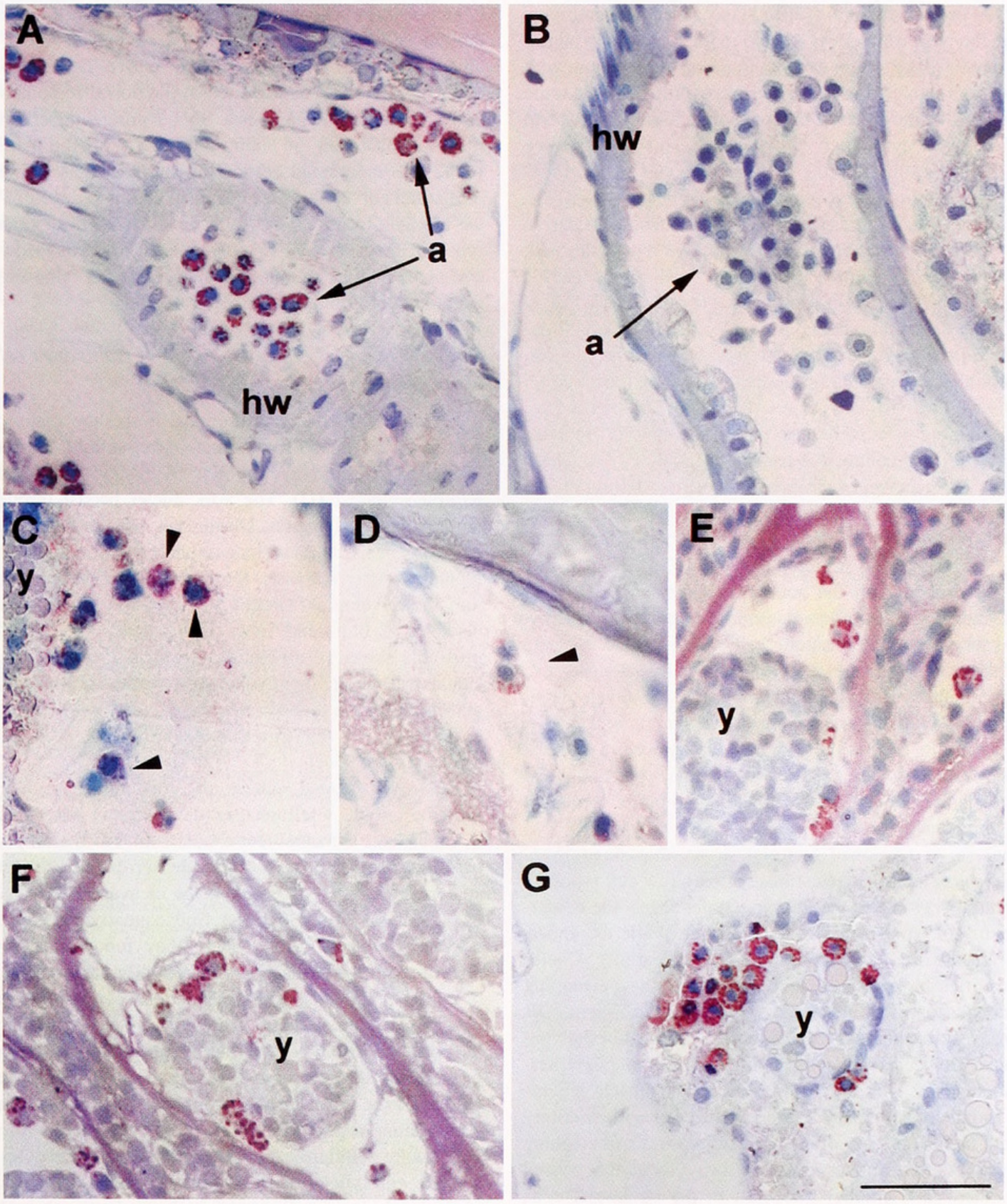

Figure 3. Amebocytes in embryos and larvae probed with the anti-coagulogen antibody and detected with *Fast* fast red. (A) Section through the heart and adjacent hemocoel regions of a trilobite larva stained with the anti-coagulogen antibody. (B) Section through the heart of a larva probed with secondary antibody only. (C, D) Amebocytes in developing hemocoel cavities of stage 18 embryos. (E) Amebocytes in a stage 20 embryo. (F, G) Amebocytes associated with distinct yolk masses in a late stage 20 embryo and a trilobite larva, respectively. a, amebocytes; hw, heart wall; y, yolk. All panels are at the same magnification, bar in G = 50 μm.

embryos or larvae examined. In stage 18 and 19 embryos, the amebocytes were always found in the developing hemocoel cavities and were usually in close association with elements of connective tissue. One possibility is that some of the cells of the developing connective tissue give rise to the first amebocytes in *L. polyphemus*. This would agree with the suggestion that the early amebocytes in *T. tridentatus* originate from mesenchymal cells (Liang *et al.*, 1990).

A second possibility is that the earliest amebocytes derive from yolk nuclei that cellularize and move out of the yolk mass into the developing hemocoel. Kishinouye (1893) suggested such an origin for amebocytes in embryos of *L. longispina* (*T. tridentatus* in current nomenclature [Sekiguchi, 1988]). Once the amebocytes have entered the cavities, they could associate with the connective tissue, forming foci in which cell division gives rise to additional amebocytes. Some dividing cells were seen within the cavities, in association with the connective tissue (Fig. 1B, F, G). These mitotic cells may represent pro-amebocytes that retain the ability to divide. It is generally accepted that mature amebocytes do not divide (Yeager and Tauber, 1935; Armstrong, 1985). As no dividing cells were observed in the embryos probed with the anti-coagulogen antibody or in those stained with iron hematoxylin, it remains to be determined whether the few mitotic cells that were observed in Giemsa-stained specimens represent pro-amebocytes.

Why amebocyte production begins at a later developmental stage in *L. polyphemus* than in *T. tridentatus* is not clear. One possibility is that the rate of development of internal structures and tissues relative to external morphological features is accelerated in *T. tridentatus* embryos compared to *L. polyphemus* embryos.

Analysis of sectioned material shows that stage 18 *L. polyphemus* embryos consist of relatively few cell types. In the ventral region and the growing appendages, the epidermal cells are typically columnar in shape, while the underlying mesodermal cells are flattened in appearance. Coelomic cavities have formed within some of the developing appendages, and within these we occasionally observe cells that have a fibroblast-like appearance. Also within the ventral plate are occasional cells having dark-staining cytoplasmic inclusions. These cells are most likely muscle precursors. In later stage embryos and larvae, cells with similarly stained inclusions are often seen adjacent to developing muscles. As one moves dorsally away from the ventral plate, there is a gradual transition in the epidermal cells from columnar through cuboidal to a flattened appearance. Similarly, the mesodermal cell layers in the dorsal region are very thin flat sheets that are often difficult to discern. By stage 18, the extension of the mesoderm over the central yolk mass appears to be complete or nearly so. The central region of the embryo is filled with yolk, within which are distributed numerous yolk nuclei. No evidence of internal organs is seen at this stage, although hemocoel cavities have begun to form. The cavities are located between the mesodermal cell layers, and within the cavities granular proamebocytes and elements of connective tissue are frequently observed. Also located in the dorsal regions of the embryo are cells that appear to be producing chitin-like material (based on the staining properties of the material). Finally, as mentioned before, the lateral organs, composed of distinct goblet-shaped cells, have begun to develop. Thus we are able to identify at most seven to eight distinct cell types in the stage 18 *L. polyphemus* embryos. How this compares with *T. tridentatus* embryos at the same stage of development remains to be determined.

Do the yolk nuclei represent a pool of multipotent cell precursors?

In contrast to many arthropods, horseshoe crabs retain significant numbers of yolk nuclei after cellular blastoderm formation (Kishinouye, 1893; Kingsley, 1892, 1893; Kimble *et al.*, 2002). The yolk nuclei persist throughout embryonic development. During the mid- to late stages of embryogenesis, some yolk nuclei probably function as vitellophages. After hatching, the residual yolk is incorporated into the developing midgut and digestive diverticulum, a network of blind-end caeca that extends throughout the prosoma. We have previously shown that some of the residual yolk nuclei cellularize to form the columnar epidermal lining of the digestive caeca, while others form a layer of flattened cells that surround the individual caeca (Kimble *et al.*, 2002).

In most arthropod species, the yolk nuclei or yolk cells function only as vitellophages, degenerating before the end of embryonic development (Anderson, 1973; Campos-Ortega and Hartenstein, 1997). In the terrestrial chelicerates, spiders and scorpions, most of the cleavage nuclei participate in blastoderm formation. Subsequently some cells repopulate the yolk mass, where they function as vitellophages. Eventually the vitellophages migrate to the surface of the yolk mass and form the endoderm epithelium (Anderson, 1973). Thus, a role for the yolk nuclei or vitellophages in formation of the gut endoderm appears to be common to most if not all chelicerates. However, participation in formation of the mesodermal components of the gut is apparently unique to the Xiphosura. If, as suggested here, some yolk nuclei cellularize and differentiate as amebocytes during late embryogenesis, it would suggest that retention of large numbers of yolk nuclei in horseshoe crab embryos provides the embryos with a pool of undetermined nuclei that can be utilized in a variety of distinct tissues during development.

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