The two origins of hemocytes in *Drosophila*

Anne Holz*, Barbara Bossinger, Thomas Strasser, Wilfried Janning and Robert Klapper†

Institut für Allgemeine Zoologie und Genetik der Westfälischen Wilhelms-Universität, Schloßplatz 5, 48149 Münster, Germany
*Present address: Institut für Allgemeine und Spezielle Zoologie, Allgemeine Zoologie und Entwicklungsbiologie, Justus-Liebig-Universität Giessen, Stephanstrasse 24, 35390 Giessen, Germany
†Author for correspondence (e-mail: klapper@uni-muenster.de)

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Summary

As in many other organisms, the blood of *Drosophila* consists of several types of hemocytes, which originate from the mesoderm. By lineage analyses of transplanted cells, we specified two separate anlagen that give rise to different populations of hemocytes: embryonic hemocytes and lymph gland hemocytes. The anlage of the embryonic hemocytes is restricted to a region within the head mesoderm between 70 and 80% egg length. In contrast to all other mesodermal cells, the cells of this anlage are already determined as hemocytes at the blastoderm stage. Unexpectedly, these hemocytes do not degenerate during late larval stages, but have the capacity to persist through metamorphosis and are still detectable in the adult fly.

A second anlage, which gives rise to additional hemocytes at the onset of metamorphosis, is located within the thoracic mesoderm at 50 to 53% egg length. After transplantation within this region, clones were detected in the larval lymph glands. Labeled hemocytes are released by the lymph glands not before the late third larval instar. The anlage of these lymph gland-derived hemocytes is not determined at the blastoderm stage, as indicated by the overlap of clones with other tissues. Our analyses reveal that the hemocytes of pupae and adult flies consist of a mixture of embryonic hemocytes and lymph gland-derived hemocytes, originating from two distinct anlagen that are determined at different stages of development.

Key words: *Drosophila*, Hemocytes, Clonal analysis, Cell lineage, Transplantation, Blood, GFP, Lymph gland, Macrophage, Hematopoiesis

Introduction

The hemolymph of *Drosophila* contains hemocytes that either circulate freely through the body cavities or are sessile, being associated with various tissues and organs. Hemocytes are responsible for the phagocytosis of apoptotic cells and thus are important for embryonic tissue formation as well as organ remodelling during metamorphosis (Abrams et al., 1993; Franc et al., 1996; Franc, 1999; Hartenstein and Jan, 1992; Tepass et al., 1994). Furthermore, hemocytes play a crucial role in immunological processes (reviewed by Hoffmann and Reichhart, 2002; Lavine and Strand, 2002). In response to an infection, they engulf and melanize foreign material and synthesize and secrete antimicrobial peptides (Braun et al., 1998; Ramet et al., 2002; Sorrentino et al., 2002).

Recent analyses have revealed both genetic and functional similarities between several aspects of insect and mammalian hematopoiesis (reviewed by Franc, 2002; Hoffmann et al., 1999; Traver and Zon, 2002). In both systems, the family of GATA transcription factors (Serpent/GATA), their co-factors (U-shaped/FOG), the AML1 domain family transcription factors (Lozenge/Runx1) (reviewed by Fossett and Schulz, 2001) as well as Notch signaling (Duvic et al., 2002) are essential for blood-cell determination and differentiation into specific cytotypes. In *Drosophila*, hematopoiesis takes place at two different stages of ontogenesis: a first population of hemocytes arises from the head mesoderm during early embryogenesis, followed by a second population that derives from the mesodermal lymph glands at a later stage of development (Traver and Zon, 2002).

During embryogenesis of *Drosophila*, a proportion of the mesodermal cells originating from the head region migrate along specific pathways and subsequently disperses throughout the body (Hartenstein and Jan, 1992; Tepass et al., 1994). These embryonic hemocytes (EH) either differentiate into small spherical cells with phagocytic capacities, so-called plasmatocytes, or into crystal cells that are involved in the melanization of pathogens (Alfonso and Jones, 2002; Franc et al., 1996; Franc, 1999; Lanot et al., 2001; Lebestky et al., 2000). The *Drosophila* GATA homolog *serpent* (*srp*) is expressed in all embryonic hemocyte precursors and is also required for the development of plasmatocytes and crystal cells (Rehorn et al., 1996; Sam et al., 1996).

In larvae, five major types of hemocytes have been described (Lanot et al., 2001; Rizki, 1957; Rizki, 1978; Rizki and Rizki, 1980; Rizki and Rizki, 1984; Rizki and Rizki, 1992; Rizki et al., 1980; Shrestha and Gateff, 1982): (1) plasmatocytes, which make up to 95% of the circulating hemocytes; (2) podocytes, which develop from plasmatocytes at the end of the third larval instar and are characterized by their pseudopodia-like extensions; (3) crystal cells; (4) lamellocytes, large flat cells that presumably differentiate from plasmatocytes in response to parasitic infections; and (5) small sessile cells, found in segmental clusters on the integument. It has been proposed that the larval hemocytes are produced and released by the lymph glands (Bairati, 1964; Rizki, 1978; Rizki and Rizki, 1980; Rizki and Rizki, 1984; Shrestha and Gateff, 1982; Stark and Marshall, 1930). However, as the release of blood cells by the lymph gland into the hemocoel or dorsal vessel has never been directly observed prior to late larval or early pupal stages (Lanot et al., 2001), the function of the lymph gland as a source of larval hemocytes has been questioned by several authors (el Shatoury, 1955; Srdic and Reinhardt, 1980).

The lymph glands, which are of mesodermal origin, are formed along the anterior part of the dorsal vessel during embryogenesis (Campos-Ortega and Hartenstein, 1997; Poulson, 1945; Poulson, 1950; Rugendorff et al., 1994; Stark and Marshall, 1930). In the larva, four to six pairs of lymph gland lobes are located lateral to the tube of the dorsal vessel (el Shatoury, 1955; Rizki, 1978). Whereas the anteriormost pair of the larval lymph gland lobes contains active secretory cells, plasmatocytes, crystal cells and undifferentiated prohemocytes, the posterior lobes predominantly contain prohemocytes (Lanot et al., 2001). As the lymph glands are eliminated at metamorphosis (Lanot et al., 2001; Robertson, 1936) and there is no evidence for an imaginal hematopoietic organ, it is commonly believed that the lymph gland-derived hemocytes (LGH) persist through metamorphosis.

However, up to now it was not possible to trace the different hemocyte populations throughout development. In this study, we performed transplantations of genetically labeled cells to follow the hemocytes from their formation up to the adult fly. Using this approach, we could show that the EH are already determined at the blastoderm stage. Both EH and LGH persist through metamorphosis and together represent the cellular components of the adult blood.

Materials and methods

Fly stocks

As donors for transplantation experiments, we used several strains detailed below. The enhancer trap strain ah92 with the genotype *P[lArB]; P[lArB] ry*^{506} (2; 3) is viable when homozygous and shows a strong nuclear β-galactosidase expression in all tissues from late embryonic stages onward. The progeny of the cross between *GAL4daG32* (Wodarz et al., 1995) and *UASlacZ4-1-2* (Brand and Perrimon, 1993) exhibits strong β-galactosidase expression in all tissues throughout the entire life cycle. The strain *Gal4daG32; UAS-GFP.S65T* strongly expresses GFP throughout all developmental stages and was employed for the in vivo examination of the transplanted cells and their progeny. It was constructed from the two lines *GAL4daG32* (Wodarz et al., 1995) and *UAS-GFP.S65T* (B. Dickson, unpublished). The strain Cg9 is deficient for the *lacZ-1* gene (Knipple and MacIntyre, 1984) and served as recipient in all transplantation experiments. The strains *UASlacZ4-1-2* and *UAS-GFP.S65T* were obtained from the Bloomington *Drosophila* Stock Center, the strain *GAL4daG32* was a generous gift from Elisabeth Knust.

Transplantation experiments

For cell lineage analyses, single cells were transplanted at the cellular blastoderm stage following the transplantation technique of Meise and Janning (Meise and Janning, 1993). In addition to single-cell transplantations, transplantations of five to ten cells were carried out. The resulting clones of β-galactosidase-expressing cells were examined at the third larval instar or in the adult fly. The preparation and staining procedure was carried out according to Holz et al. (Holz et al., 1997). Living embryos and third-instar larvae were examined for GFP expression and raised to adulthood. For detailed examination of GFP expression, larvae and adult flies were dissected in transplantation solution (Klapper, 2000; Meise and Janning, 1993). An Olympus inverse microscope CK40 equipped with an EGFP filter set (AHF Analysentechnik) and a video enhancement system were used for fluorescence analysis.

Immunohistochemical staining

To identify hemocytes, which are specified by their characteristic expression of peroxidasin, we carried out double labeling. After the X-gal staining that was employed to highlight the descendants of the transplanted cell, we additionally performed an immunohistochemical staining using a mouse anti-peroxidasin antibody (Nelson et al., 1994) at a 1:10 dilution (anti-peroxidasin was kindly provided by Rolf Reuter). Secondary antibodies conjugated to Biotin (Jackson ImmunoResearch) were used at 1:200 dilutions. Biotinylated secondary antibodies were detected by using the Vectastain Elite ABC kit and HRP/diaminobenzidine (DAB) reaction. The immunohistochemical staining was carried out following standard protocols.

Results

The localization of the embryonic hemocyte primordium at blastoderm stage

In order to localize the anlage of the embryonic hemocytes (EH) and to analyze the cell lineage of these cells, we carried out homotopic single cell transplantations to generate position-specific clones at the blastoderm stage. By the use of donors that ubiquitously express β-galactosidase, the transplanted cell and its complete progeny can be detected at any developmental stage within the host individual.

Because it was shown that the EH originate from the head mesoderm region, we performed homotopic transplantations between 51 and 93% EL (EL, egg length; 0% EL, posterior pole) and 0 to 30% VD (VD, ventrodorsal; 0% VD, ventral midline). Sixty-five percent EL corresponds to the prospective position of the cephalic furrow, dividing the head from the trunk mesoderm, whereas 85% EL represents the anterior border of the mesoderm at the blastoderm stage. The mesodermal anlage extends dorsoventrally from the ventral midline to about 30% VD. To minimize possible heterotopic effects, we examined only transplantations in which the sites of cell removal and integration differ by no more than 5% EL. This corresponds to about five cell diameters at the blastoderm stage.

Of 2458 homotopic single-cell transplantations carried out between 51 and 93% EL, we detected 452 mesodermal and 33 endodermal clones in 3rd instar larvae (Table 1). In addition to these clones, we found some ectodermal clones at each position within the transplantation region. These ectodermal clones derived from transplantations of donor cells originating from the lateral border of the mesoderm anlage between 10 and 30% VD and were not further considered in this analysis. Besides 374 clones in mesodermal tissues, such as the fat body, somatic musculature and visceral musculature, we also detected 78 clones in hemocytes.

Hemocyte clones are easily distinguishable from all other transplantation clones within mesodermal, ectodermal or endodermal tissues by their specific cell morphology, scattered distribution (Fig. 1A) as well as by their characteristic expression of peroxidasin (Nelson et al., 1994) (Fig. 1B). Labeled hemocytes of individual clones were randomly mixed with clonally non-related additional hemocytes and found widely dispersed in third instar larvae (Fig. 1A-C). This scattered distribution demonstrates the independent mobility of sister cells. Most hemocytes were detected in clustered groups in the abdominal segments (Fig. 1A,B), more often located in dorsal than in ventral regions. Smaller fractions of labeled

Table 1. Homotopic single-cell transplantations in the head mesoderm between 70 and 80% EL frequently result in hemocyte clones

Transplantation region	93-81% EL	80-70% EL	69-51% EL	Total number
Transplantations	251	759	1448	2458
All mesodermal clones	29	111	312	452
Hemocytes	1	77	0	78
Endodermal clones	27	6	0	33

EL, egg length; 0% EL, posterior pole.

hemocytes were frequently detected in the lateral region of the thoracic segments (data not shown) and in most cases several hemocytes were attached specifically to the epithelium of the eye-antenna disc (Fig. 1C). In general, hemocyte clones are very large and consist of up to about 300 marked descendants of the transplanted cell, whereas 16 labeled hemocytes represent the smallest clones. This reveals that the transplanted cells performed four to nine postblastodermal mitoses up to the end of the 3rd larval instar.

Seventy-seven out of 78 hemocyte clones (99%) originate from a region restricted to 70-80% EL (Tables 1 and 2), revealing a sharply delimitated hemocyte primordium. Anteriorly and posteriorly, this region is flanked by mesoderm giving rise to somatic muscles (Fig. 1E). Thus, we were able to map the EH anlage precisely within the head mesoderm region.

The anterior border of the head mesoderm is located at 85% EL. Anterior to this border only clones contributing to the anterior midgut were detected. Within these clones, larval and imaginal cells of the anterior midgut epithelium frequently overlapped (Fig. 1D). This demonstrates that the cells of the endoderm anlage are not determined towards their prospective larval or imaginal cell fate at the blastoderm stage.

The embryonic hemocytes are already determined at the blastoderm stage

Previous transplantation experiments within the thoracic and abdominal mesoderm revealed that the descendants of a single transplanted cell can give rise to as many as four different mesodermal tissues (Beer et al., 1987; Holz et al., 1997; Klapper et al., 1998). So far, there is no evidence for a tissue-specific determination within the mesoderm prior to the second postblastodermal mitosis. However, none of the 72 hemocyte clones overlapped with other mesodermal derivatives, suggesting that the embryonic hemocyte (EH) anlage might already be determined at the blastoderm stage.

In order to test this possibility, we carried out heterotopic transplantations at the blastoderm stage. Single cells were transplanted either from outside the EH anlage into the EH anlage (Table 2B) or vice versa (Table 2C,D). Of 334 heterotopic single-cell transplantations into the EH primordium, 59 resulted in mesodermal clones (Table 2B). None of these clones contributed to hemocytes.

By contrast, transplantations from the EH anlage into the adjacent regions of the mesoderm frequently gave rise to hemocyte clones (Table 2C,D). These clones are indistinguishable from homotopic transplantation clones: the marked cells intermingle with other (unlabelled) hemocytes, exhibit the same morphology and colonize identical positions in third instar larvae (Fig. 2A,B). Taken together, these results demonstrate that the hemocytes are already determined at the blastoderm stage. A determination of other mesodermal cells towards an EH fate is not possible from the blastoderm stage onwards.

The embryonic hemocytes persist through metamorphosis

The use of β-galactosidase as clone marker allows the detection

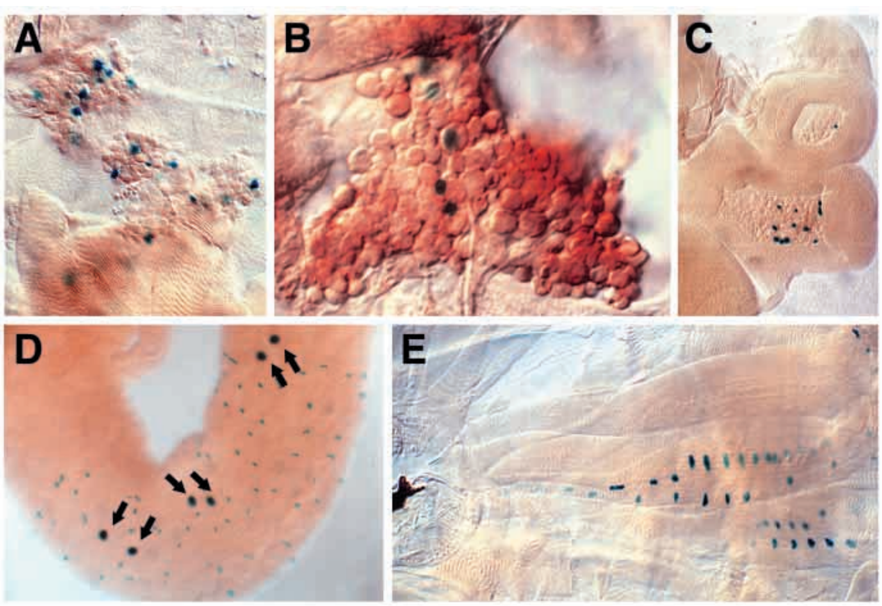
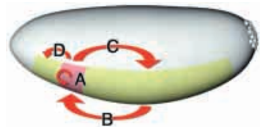


Fig. 1. The embryonic hemocyte (EH) anlage is restricted to 70-80% EL. Clones in third instar larvae after homotopic single-cell transplantation within the head mesoderm. (A) Clone fraction labelling several hemocytes situated in the abdomen of a larva resulting from a homotopic transplantation at 75% EL. (B) Besides their morphology, the labeled cells (blue nuclei) were identified as hemocytes by their characteristic expression of peroxidasin (brown cells). (C) In many cases, labeled hemocytes were detected on the eye-antenna disc of dissected third instar larvae. (D) Transplantation anterior to 85% EL gave rise to endodermal clones. The midgut clone consists of six larval cells (arrows) and many additionally labeled imaginal cells and was obtained after a homotopic single cell transplantation at 89% EL. (E) The EH anlage is embedded into the mesodermal anlage, giving rise to somatic muscles, like the clone in the large head-retractor muscles, deriving from a transplantation at 68% EL.

Table 2. The localization and determination of the hemocyte anlage at the blastoderm stage revealed by homo- and heterotopic single-cell transplantations



		Origin of the transplanted cell in % EL		
		93-81	80-70	69-20
Integration of the transplanted cell in % EL	93-81	One hemocyte + 28 other mesodermal clones*	(D) 22 hemocyte + 11 other mesodermal clones	–
	80-70	No hemocyte + 7 other mesodermal clones	(A) 77 hemocyte + 34 other mesodermal clones*	(B) No hemocyte + 59 other mesodermal clones
	69-20	–	(C) 12 hemocyte + 9 other mesodermal clones	No hemocyte + 312 other mesodermal clones*

n=539 homo- and heterotopic mesodermal clones; EL, egg length.
*Results from homotopic transplantations.

of a given clone only once per individual development because the specimen has to be dissected and fixed in order to visualize the reporter gene expression. To trace individual clones through different stages of development, we employed a ubiquitously GFP-expressing donor line. Resulting clones were examined in stage 17 embryos, third instar larvae, pupae and adult flies [stages according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997)].

Two-hundred and twenty-nine homotopic single-cell transplantations were carried out within the hemocyte anlage between 70 and 80% EL. In 101 of these a hemocyte clone was identified in stage 17 embryos (Fig. 3A). Up to 16 hemocytes per embryo were labeled, indicating that the transplanted cell performed a maximum of four postblastodermal mitoses. Of the 101 embryos showing a hemocyte clone, 72 survived until the third larval instar. In 56 of these larvae, the previously observed hemocyte clone was re-detected (Fig. 3B,C). At this stage of development, the clone sizes varied from 50 to 300 labeled hemocytes. This reveals that the embryonic hemocytes (EH) performed up to five additional mitoses during larval development. On the basis of their morphological characteristics (spherical shape, presence of filamentous extensions) and their motility, the observed hemocytes were classified as plasmatocytes and podocytes (data not shown). We also observed clusters of sessile hemocytes, possibly resembling the class of sessile hemocytes described by Lanot et al. (Lanot et al., 2001).

Twenty-one of these 56 larvae also survived metamorphosis. In all of them, labeled hemocytes were detected again (Fig. 3D). Owing to the non-transparency of the adult cuticle, an accurate counting of the hemocytes was not feasible. In dissected flies, the clone sizes ranged from about 20 to 50 labeled hemocytes, which were classified as plasmatocytes and podocytes (data not shown). These results demonstrate that at least a fraction of the EH persists through all stages of development. Moreover, labeled hemocytes were not only visible in newly hatched flies but still detectable 14 days after emergence. Thus, the EH represent an enduring component of the adult blood.

The lymph glands release hemocytes at the onset of metamorphosis

Several analyses, mainly based on morphological observations, indicated that the lymph glands produce and release hemocytes found in larvae (Bairati, 1964; Rizki, 1978; Shrestha and Gateff, 1982; Stark and Marshall, 1930). However, none of the hemocyte clones originating from the head mesoderm labeled parts of the lymph glands at any time of development. Furthermore, after transplantation of several thousand single mesodermal cells to sites outside the embryonic hemocyte (EH) anlage described above, we never detected clones contributing to hemocytes in third instar larvae (Holz et al., 1997; Klapper, 2000; Klapper et al., 2001; Klapper et al., 1998; Klapper et al., 2002). Besides other mesodermal clones (data

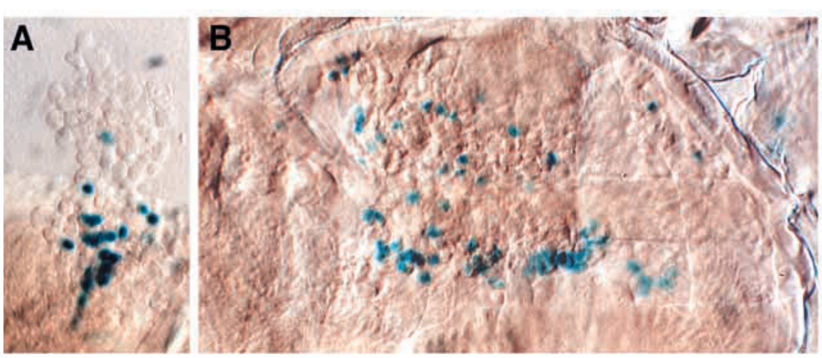


Fig. 2. Heterotopic transplantation reveals the determination of hemocytes at the blastoderm stage. Clones labelling hemocytes in third instar larvae after heterotopic transplantation of single cells from 70-80% EL into the abdominal mesoderm anlage. (A) Fraction of labeled hemocytes intermingled with additional, non-labeled hemocytes derived from a transplantation of a cell originating from 76% EL to 43% EL. (B) Like labeled hemocytes from homotopic transplantations, the hemocytes derived from heterotopic transplantations are also found to be widely dispersed in larvae, as evidenced by this clone fraction in the abdomen.

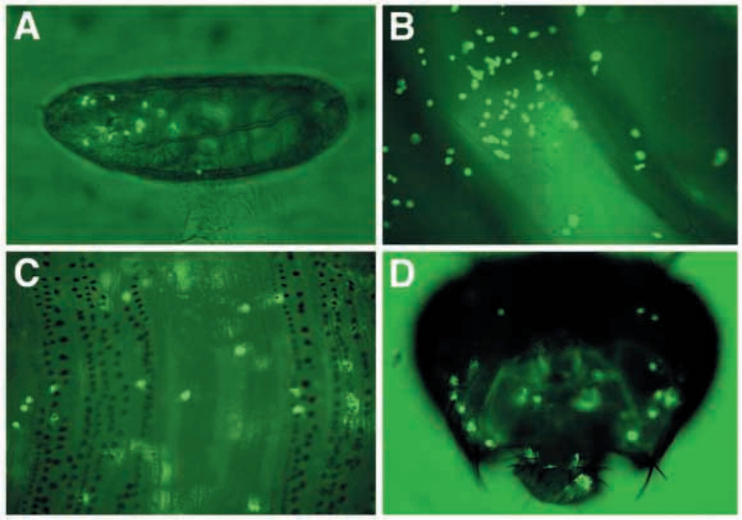


Fig. 3. Embryonic hemocytes (EH) persist through all stages of development. Individual clone after homotopic transplantation of a single GFP-labeled cell at 74% EL at different developmental stages. (A) Several scattered GFP-expressing cells are detectable in a stage 17 embryo. (B) In the third instar larva many dispersed hemocytes either circulate or are attached to the integument. (C) Hemocytes are also detectable in the prepupa. (D) EH persist through metamorphosis, as revealed by the presence of many GFP-expressing cells in the head of the 14-day-old fly.

not shown), we obtained only three clones contributing to the larval lymph glands. In all three cases, the transplantations were carried out between 50 and 53% EL and resulted in clones labelling one of the lymph gland lobes almost entirely. This indicates that only a few progenitor cells give rise to a lymph gland lobe. Two of the clones additionally labeled somatic muscles (Fig. 4A), revealing that the lymph gland – in contrast to the hemocytes originating from the head mesoderm – is not determined at the blastoderm stage. In none of these three clones were hemocytes outside the lymph gland labeled.

In order to increase the incidence of lymph gland clones, we performed a series in which up to 10 GFP-expressing cells were transplanted at the same time. Out of 290 transplantations within a region between 45 and 60% EL, 196 individuals survived to the early third larval instar and were examined for clones in vivo. Besides other labeled mesodermal tissues, in 22 cases we detected clones contributing to the lymph glands, which confirms that they arise from about 50% EL (Fig. 4B,C). However, none of these clones contributed to hemocytes outside the lymph glands when examined at the early third larval instar.

Nine out of the 22 larvae with a lymph gland clone were examined repeatedly until the onset of puparium formation (Fig. 4D-F). In seven of these larvae, labeled hemocytes became visible at the late third larval instar, directly prior to pupation. This shows that the lymph glands give rise to hemocytes, but that the release does not take place prior to the onset of metamorphosis. Of the seven specimens, three survived to adulthood and were examined once again. In all three individuals, large hemocyte clones consisting of sessile as well as circulating GFP-expressing cells were detected (Fig. 4G). On the basis of morphological criteria, the lymph gland derived hemocytes (LGH) in adult flies were not distinguishable from the persisting EH. Owing to the degradation of the lymph glands during metamorphosis (Robertson, 1936), the labeling within this tissue were not redetected.

Taken together, our observations indicate that the embryonic

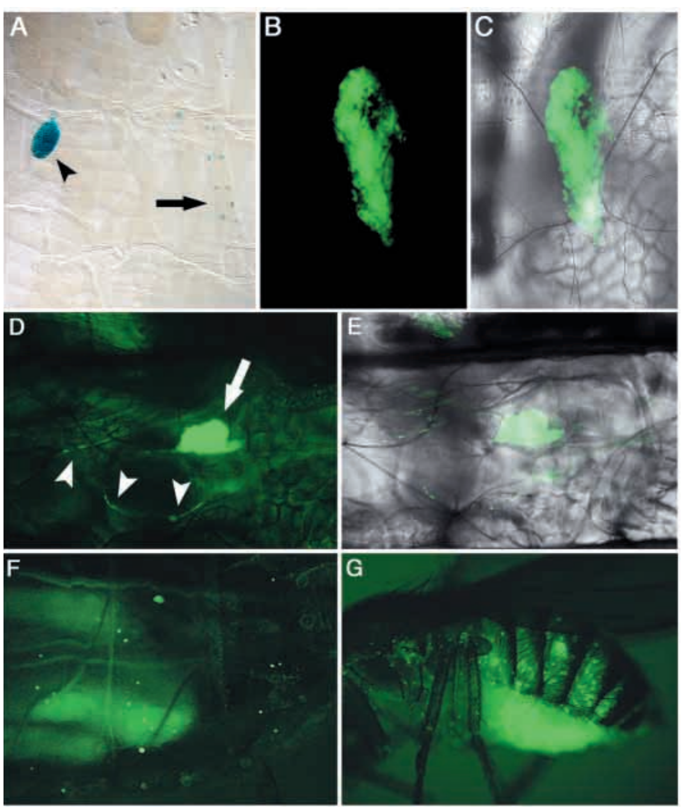


Fig. 4. The lymph glands release hemocytes at the onset of metamorphosis. (A-G) Clones after homotopic transplantations of a single cell (A) or about 10 cells (B-G) between 50 and 55% EL. (A) Lymph gland clone (arrowhead) overlapping with dorsal somatic muscles (arrow) in a third instar larva. (B,C) GFP labelling in a lymph gland lobe of a third instar larva (B, epifluorescence; C, merged with bright-field image). No labeled hemocytes were detected outside of the lymph gland. (D,E) Labelling of a lymph gland lobe (arrow) and longitudinal visceral muscles (arrowheads) in the third instar larva (D, epifluorescence; E, merged with bright-field image). (F) The same specimen as in D at the prepupa stage: several hemocytes are detected either circulating within the hemolymph or sessile on the integument. (G) After metamorphosis, many labeled hemocytes are visible in the adult fly (same specimen as in D and F).

hemocytes as well as the lymph gland hemocytes persist through metamorphosis. Thus, the blood of the adult fly is composed of two subpopulations of hemocytes that have two different spatial and temporal origins.

Discussion

The transplantation of single genetically marked cells is a versatile tool for cell lineage analyses and fate mapping studies (Prokop and Technau, 1993). As this technique allows clones to be generated at specific regions of the embryo at a defined stage, and the descendants of the transplanted cell to be followed throughout development, important information about determination events, developmental capacities and cell lineage relationships can be obtained. We used this approach to localize and characterize the primordia giving rise to hemocytes in *Drosophila*.

The embryonic hemocytes are already determined at the blastoderm stage

It has previously been shown that the origin of the embryonic hemocytes (EH) can be traced back to the head mesoderm of late stage 11 embryos by morphological criteria (Tepass et al., 1994). Owing to the fact that *srp* is expressed in a narrow stripe within the cephalic mesoderm at the blastoderm stage and that a loss of *srp* function leads to a complete loss of embryonic hemocytes, the primordium of the EH was referred to the respective expression domain (Rehorn et al., 1996). By homotopic single-cell transplantations we were able to restrict the anlage to a sharply delimitated region located at 70 to 80% EL within the mesoderm (Fig. 5), exactly corresponding to the cephalic expression domain of *srp*. The fact that none of the EH clones overlapped with other tissues indicated that the hemocytes are already determined at the blastoderm stage. This was confirmed by heterotopic transplantations from the EH anlage into the abdominal mesoderm, which also gave rise to hemocytes. As mesodermal cells transplanted into the EH anlage are not determined into EH, the determining factor is not able to induce a hemocyte fate within these cells and seems to function cell-autonomously. A good candidate for such a factor is Srp, a member of the GATA-binding transcription factor family. However, as *srp* is also expressed in many other tissues that do not give rise to hemocytes (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996), there must be additional genes that lead to a determination of the EH at the blastoderm stage. The early determination of the EH is quite unusual, as all other mesodermal tissues analysed so far – including the anlage of the LGH – were not restricted to a tissue-specific fate prior to the second postblastodermal mitoses (Beer et al., 1987; Holz et al., 1997; Klapper et al., 1998). This might be a developmental adaptation of the EH, which at stage 12 are already differentiated into functional macrophages and are responsible for the removal of apoptotic cells within developing tissues (Abrams et al., 1993; Franc et al., 1996; Franc, 1999; Hartenstein and Jan, 1992; Tepass et al., 1994).

The embryonic hemocytes persist through metamorphosis while additional hemocytes are released by the lymph glands during pupation

It is commonly believed that in *Drosophila* during larval development the EH population is entirely replaced by hemocytes that have been released by the larval lymph glands. However, we were able to trace hemocytes originating from the head mesoderm through all stages of development until 14-day-old adult flies. Lanot et al. (Lanot et al., 2001) described that the number of hemocytes progressively rises during larval life, from less than 200 to more than 5000 per individual. Our cell lineage analyses unambiguously demonstrate that this increase is due to postembryonic proliferation of the EH. We also determined the contribution of the lymph glands to the hemocyte population by means of cell lineage analyses. In contrast to earlier descriptions, our studies reveal that the lymph glands do not release blood cells into the hemocoel during all larval stages but exclusively at the end of the third larval instar, as also proposed by Lanot et al. (Lanot et al., 2001). With the onset of metamorphosis, additional hemocytes are released from the lymph glands. Although the lymph glands do not persist through metamorphosis (Lanot et al., 2001; Robertson, 1936), the marked hemocytes released by the labeled lymph glands are still detectable in adult flies. Hence, all hemocytes found throughout larval life originated solely from the EH anlage, whereas the pupal and imaginal blood is made up of two different populations: EH and LGH.

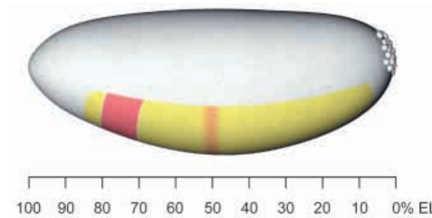


Fig. 5. Blastoderm fate map of the embryonic hemocytes (EH) and lymph gland-derived hemocytes (LGH). Within the mesoderm anlage (yellow), which is represented by the ventral region of the blastoderm embryo reaching from 85 to 5% EL, we could identify two anlagen giving rise to hemocytes (red). The EH anlage, which is already determined at the blastoderm stage, is embedded into the head mesoderm and restricted to 70-80% EL. The anlage of the LGH is located at 50-55% EL. Cells giving rise to the lymph glands, and therefore to the LGH, are not determined towards a tissue-specific fate prior to the second postblastodermal mitosis.

The two origins of hemocytes

Previous studies, as well as our cell lineage analyses, reveal that the two populations of hemocytes share many functional, morphological and genetic similarities. In both cases, the determination of hemocytes depends on *srp* (Lebestky et al., 2000; Rehorn et al., 1996), while the specification towards the distinct blood cell types is induced by the expression of *lozenge* (*lz*) (Lebestky et al., 2000), *glia cells missing* (*gcm*) (Bernardoni et al., 1997) and the *gcm* homolog *gcm2* (Alfonso and Jones, 2002). Both EH and LGH differentiate into podocytes, crystal cells and plasmatocytes (Lanot et al., 2001). Hemocytes of both populations have the capability to adopt macrophage characteristics. However, despite all similarities, the history of the two populations is quite different, as they originate from two different mesodermal regions and are determined at different developmental stages. In view of the fact that the lymph glands do not release hemocytes before the onset of metamorphosis under nonimmune conditions, all hemocytes found in the larval hemocoel represent EH. This was not taken into account in several genetic analyses of embryonic and larval hemocytes. Thus, it may be possible that some of these data have to be reconsidered, taking into account that not lymph gland derived larval hemocytes were studied, but EH during larval development.

The many similarities between EG and LGH raise the question why there are two populations at all. As also observed in many other studies, we noted a massive release of hemocytes by the lymph glands just at the onset of pupation. Lanot et al. (Lanot et al., 2001) could show that the lymph glands additionally have the capacity to differentiate and release a special type of hemocytes, the lamellocytes, under immune conditions even before the onset of metamorphosis. Thus, because under nonimmune conditions the lymph glands do not release any cells before the onset of pupation, it might be their primary role to provide a reservoir of immune defensive hemocytes. The massive apoptosis and accumulation of cell debris might be a secondary trigger to stimulate proliferation and release of the lymph gland hemocytes.

To study *Drosophila* hematopoiesis, the knowledge of which hemocyte population is present at different stages of development is pivotal. To avoid misunderstandings associated with the confusing term 'larval hemocytes', which has been used to describe all kinds of hemocytes in postembryonic development, we propose the use of the terms embryonic hemocytes (EH) and lymph gland hemocytes (LGH).

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