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Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*

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Abstract

Hemocytes are an essential component of the mosquito immune system but current knowledge of the types of hemocytes mosquitoes produce, their relative abundance, and their functions is limited. Addressing these issues requires improved methods for collecting and maintaining mosquito hemocytes in vitro, and comparative data that address whether important vector species produce similar or different hemocyte types. Toward this end, we conducted a comparative study with *Anopheles gambiae* and *Aedes aegypti*. Collection method greatly affected the number of hemocytes and contaminants obtained from adult females of each species. Using a collection method called high injection/recovery, we concluded that hemolymph from *An. gambiae* and *Ae. aegypti* adult females contains three hemocyte types (granulocytes, oenocytoids and prohemocytes) that were distinguished from one another by a combination of morphological and functional markers. Significantly more hemocytes were recovered from *An. gambiae* females than *Ae. aegypti*. However, granulocytes were the most abundant cell type in both species while oenocytoids and prohemocytes comprised less than 10% of the total hemocyte population. The same hemocyte types were collected from larvae, pupae and adult males albeit the absolute number and proportion of each hemocyte type differed from adult females. The number of hemocytes recovered from sugar fed females declined with age but blood feeding transiently increased hemocyte abundance. Two antibodies tested as potential hemocyte markers (anti-PP06 and anti-Dox-A2) also exhibited alterations in staining patterns following immune challenge with the bacterium *Escherichia coli*.

Keywords

insect; immunity; encapsulation; phagocytosis; hemocyte

The ability to isolate and identify hemocytes is essential for studies in insect cellular immunity. Hemocyte classification schemes based on morphology or a combination of morphological and functional characters have been developed for the model dipteran *Drosophila melanogaster* and selected Lepidoptera (Gupta, 1985; Brehelin and Zachary, 1986; Lackie, 1988; Ratcliffe, 1993; Gillespie et al., 1997; Lanot et al., 2001; Lavine and Strand, 2002; Irving et al., 2005; Wertheim et al., 2005). Much less is known about the types of hemocytes produced by other insects including numerous species of economic importance. Key challenges include the small size of many insects which makes collection and identification of hemocytes difficult

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due to the limited amount of hemolymph and cells present in circulation. The hemocyte types insects produce and the names they are given also sometimes differs between taxa such that classification schemes and criteria used to identify hemocytes in one group of insects may not be fully applicable to another (Lavine and Strand, 2002).

The difficulty of collecting and classifying insect hemocytes is especially apparent in vector arthropods like mosquitoes. In vivo studies indicate that hemocytes comprise an essential arm of the mosquito immune system required for phagocytosis and encapsulation of foreign targets (Christensen and Forton, 1986; Cho et al., 1998; Huang et al., 2001; Dimopoulos et al., 2001; Lanz-Mendoza et al., 2002; Hernandez et al., 1999; Levashina et al., 2001; Hillyer et al., 2003; Moita et al., 2005). Mosquito hemocytes are also important sources of signaling and effector molecules released into hemolymph (Dimopoulos et al., 2001; Hillyer et al., 2003). However, current understanding of the types of hemocytes mosquitoes produce, their relative abundance, and their functions is limited. Hemocytes from adult *Aedes aegypti* were recently classified into granulocytes, oenocytoids, adipohemocytes, and thrombocytoids on the basis of morphology, binding of selected lectins, and enzymatic activity (Hillyer and Christensen, 2002). Using strictly morphological criteria, other investigators have classified hemocytes from *Ae. aegypti* and *Culex quinquefasciatus* into plasmatocytes and oenocytoids (Andreadis and Hall, 1976; Drif and Brehelin, 1983) or have recognized multiple cell types including putative stem cells named prohemocytes (Foley, 1978; Kaaya and Ratcliffe, 1982). Far less is known about the hemocytes produced by other mosquitoes including *Anopheles gambiae* that is a major vector of human malaria. Hemocytes from anophiline mosquitoes are known to be phagocytic and have also been observed in proximity to melanotic capsules (Hernandez et al., 1999; Hernandez-Martinez et al., 2002; Lanz-Mendoza et al., 2002; Moita et al., 2005). The types of hemocytes and their function in mediating these responses, however, are unclear.

Identifying the hemocytes mosquitoes produce and understanding their functions in immunity would benefit from increased uniformity in methods for collecting cells and in the criteria used for classifying and naming different hemocyte types. It would also be valuable if comparative data collected using similar methodology were available to determine if important vector species produce similar or different hemocyte types. Toward this end, we conducted a comparative study with *An. gambiae* and *Ae. aegypti*. We first examined how different collection methods affected the number and types of hemocytes obtained from mosquitoes. We then used a combination of morphological and functional markers to classify the hemocytes present in different life stages. We conclude that both species produce three types of hemocytes that are identifiable using similar criteria.

Materials and Methods

Insects

An. gambiae (G3 strain) and *Ae. aegypti* (UGAL strain) were reared in a dedicated insectary in the Department of Entomology at the University of Georgia at ~27° C with a 16 h light: 8 h dark cycle. After hatching, larvae were reared in deionized water in shallow aluminum pans (200–250 larvae/~400 ml/tray) and fed a defined daily regimen of finely ground mixture of TetraMin Rich Mix fish food. Under these conditions, development to pupae is highly synchronous. Adults have access to 8% fructose solution, and prior to blood feeding, caged mosquitoes were starved and kept in total darkness for at least an hour.

Hemocyte collection and primary culture

Previous approaches used for collecting mosquito hemocytes include clipping the proboscis of cold anesthetized adult females (Chen and Lawrence, 1987; Chun et al., 2000; Abraham et al., 2005) and displacement perfusion (Beerntsen and Christensen, 1990). We compared these

approaches to two other approaches we developed and named low and high injection/recovery. In the low injection/recovery method, adult *An. gambiae* and *Ae. aegypti* were cold anesthetized on ice for 15 minutes followed by injection of 8–10 μ l of 60% Schneider's medium (Sigma), 10% fetal bovine serum (FBS) (Hyclone) and 30% citrate buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, buffer pH 4.5) (vol/vol) between the last two abdominal sclerites using a glass needle mounted on a micromanipulator. Diluted hemolymph was then collected by capillary action using a clean, empty glass needle placed next to the injection site in the abdomen. In the high injection/recovery method, adults were again injected with 10–12 μ l of Schneider's: FBS: anticoagulant (60: 10: 30) and placed on ice for 20 min. We then injected 25 μ l of Schneider's: FBS: anticoagulant (60: 10: 30) into the lateral wall of the mesothorax and collected the diluted hemolymph by capillary action from the original injection site in the abdomen using a second hand-held glass needle. The diluted hemolymph from both approaches was collected in a microfuge tube on ice or placed in Teflon-lined wells on glass slides (see below). After allowing cells to settle, diluted hemolymph was removed and replaced with fresh Schneider's medium plus 10% FBS. Hemocytes were collected from three day old pupae and third instar larvae using the high injection/recovery method. Other media including Grace's insect medium, TC-100, L-15, and HyQ (Sigma, Hyclone) with or without FBS were also tested as alternatives for collecting and maintaining mosquito hemocytes in primary culture. However, none of these media improved collection or viability of mosquito hemocytes compared to Schneider's medium plus 10% FBS (data not presented).

Functional assays and immunocytochemical procedures

Fluorescent probes tested as potential markers for mosquito hemocytes are listed in Table 1. Staining conditions (buffer, pH, temperature, incubation time) were first optimized by titrating with monochlorobimane (MCB) (Tirouvanzian et al., 2004). Cells were incubated with probes diluted in Schneider's medium for 30 min at room temperature in the dark at the concentrations indicated in Table 1. Cells were washed once in medium and then held at 4° C or at room temperature before examination. Phagocytosis assays were conducted using fluorescein isothiocyanate (FITC)-conjugated *Escherichia coli* prepared as previously described (Beck and Strand, 2005). One $\times 10^3$ bacteria were injected into cold anesthetized mosquitoes. After 1 h at room temperature, hemocytes were collected using the high injection/recovery method as described above. Alternatively, hemocytes were collected from individual mosquitoes and overlaid with 1×10^3 bacteria for 1 h. We then scored the percentage of each hemocyte type that had ingested particles by counting 100 hemocytes per sample using the fluorescent quenching method (Beck and Strand, 2005). The capacity of mosquitoes to encapsulate a foreign target was tested by inserting the tip of a glass needle between the intersegmental membrane of two abdominal sclerites. The fiber was then examined for binding of hemocytes 24–48 h later.

Six antibodies generated against specific *An. gambiae* immune proteins provided by collaborators were also tested (Table 2). These were antisera to: serpin6 (SRPN6), serpin10 (SRPN10), the chitin binding serine protease SP22D, and prophenoloxidase6 (PPO6) (K. Michel and F. C. Kafatos, Imperial College); lysozyme c-1 (LYS c-1) (S. Paskewitz, Univ. Wisconsin); and a subunit of the 26S proteasome (PSMD3) previously named diphenol oxidase A2 (DOXA2) (P. Romans, Univ. Toronto; see Zheng et al., 2003). An anti-histone H1 antibody (Santa Cruz) was also used as a marker for cell nuclei in selected experiments. Hemocytes from non-immune challenged mosquitoes and mosquitoes injected 3 h earlier with *E. coli* were fixed in 4% paraformaldehyde in PBS (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM Na₂HPO₄, 0.14 mM KH₂PO₄, pH 7.3) for 15 min, rinsed with PBS and then permeabilized for 15 min in PBT (PBS plus 0.1% Triton X-100). After blocking for 1 h with 1% bovine serum albumin (BSA; fraction V, Boehringer Mannheim) in PBS (blocking solution), cells were incubated with primary antibody at the dilution indicated in Table 1. After rinsing 4x in PBT, hemocytes

were incubated with fluorescein isothiocyanate (FITC), Texas Red (TR)-conjugated secondary antibodies (1:1000) (Jackson Labs) diluted in blocking solution.

Staining for phenoloxidase activity was performed by fixing hemocytes in 4% paraformaldehyde in PBS for 15 min, rinsing with PBS, and permeabilizing in 50% methanol. After rinsing in PBS, cells were incubated with 2 mg/ml L-dopamine in PBS for 3 h (Pech et al., 1994). For detection of acid phosphatase activity, samples were incubated for 30 min in 10 mg/ml lead nitrate and 3% β -glycerophosphate in 0.05 M acetate-acetic acid, pH 5.0. Cells were then rinsed 3x in water followed by incubation for 1 min in 1.0% ammonium sulfide in water (Humason, 1972). Peroxidase activity was detected by incubating fixed cells in 500 μ g/ml diaminobenzidine (DAB) in PBS followed by addition of 0.01% hydrogen peroxide.

Microscopy, image processing, and statistical analysis

Samples were examined using a Leica TCS confocal microscope fitted with differential interference contrast (DIC) optics. Some DIC and epifluorescent images were directly captured using a digital camera (Q Capture) while others were obtained by confocal microscopy using Leica software. All captured images were exported to Adobe Photoshop as tif files for assembly of figures. Each treatment was tested against hemocytes collected independently from five or more mosquitoes of specific age or stage. Proportional data were arcsin transformed prior to analysis. The data were then analyzed by t-test or one way analysis of variance (ANOVA) using JMP 3.0 software (SAS Institute, Gary, NC) (Sall and Lehman, 1996).

Results

Collection method affects the number and types of cells recovered from adult mosquitoes

Established methods for collecting mosquito hemocytes include bleeding from a cut proboscis (Chen and Lawrence, 1987; Chun et al., 2000; Abraham et al., 2005) and perfusion whereby saline or medium is injected between the head and thorax, and diluted hemolymph is collected from an incision made in the abdomen (Beerntsen and Christensen, 1990). Mosquito hemocytes have also been observed in close proximity to trachea and other tissues in the hemocoel (Levashina et al., 2001; Moita et al., 2005). This suggests that some hemocytes are sedentary or adhere to tissues in contact with hemolymph which could also affect collection. In insects like Lepidoptera, the adhesive properties of hemocytes have been reduced during collection by using low pH, anticoagulant buffers (Mead et al., 1986; Pech et al., 1994). We therefore assessed whether use of an anticoagulant could facilitate collection of mosquito hemocytes by comparing a method we called injection/recovery to the proboscis and perfusion methods. Four day old sugar fed *An. gambiae* and *Ae. aegypti* adult females were used for these comparisons by assessing the total number of hemocytes and contaminants (cuticle, scales, and internal tissues like the fat body) collected. Live hemocytes were discriminated from contaminants by adding the vital dye propidium iodide (PI) and monochlorobimane (MCB) to each sample. MCB enters living cells and interacts with the antioxidant glutathione to produce fluorescent glutathione-S-bimane. MCB is also reported to preferentially label mammalian and insect immune cells because of their elevated glutathione levels (Tirouvanziam et al., 2004).

The high injection/recovery method yielded the most hemocytes per mosquito while cutting the proboscis yielded the fewest (Fig. 1A). We also noted that more hemocytes were recovered on average from *An. gambiae* than *Ae. aegypti* using each collection method despite their smaller size (Fig. 1A). Visual inspection of the samples revealed that the perfusion method consistently produced the largest amount of contaminants which included a mixture of cells and tissue fragments from other organs (fat body, Malpighian tubules), unidentifiable subcellular debris, and scales from the abdomen (Fig. 1B). The proboscis and low injection/recovery methods produced the least contamination while the high injection/recovery method

produced slightly more contamination that consisted primarily of subcellular debris (Fig. 1C). This level of contamination, however, was much lower than the perfusion samples and was also more easily removed by gently washing the collected hemocytes with fresh Schneider's medium. Hemocyte viability from both species using the high injection/recovery method was greater than 90% as evidenced by PI staining (data not presented). Increasing the amount of anticoagulant or reducing the amount of FBS in the solution used to collect hemocytes significantly elevated mortality and did not increase the number of hemocytes recovered (data not presented).

As previously noted, Hillyer and Christensen (2002) classified hemocytes from *Ae. aegypti* into granulocytes, oenocytoids, adipohemocytes, and thrombocytoids. Granulocytes were described as being approximately 9 μm in diameter with numerous granules in the cytoplasm. Granulocytes were also the most abundant hemocyte type, were phagocytic and bound to foreign surfaces like glass slides. Adipohemocytes were reported to be the second most abundant hemocyte type, averaging 40 μm in diameter with large nuclei and prominent lipid droplets in the cytoplasm. Oenocytoids were described as being approximately 9 μm in diameter with a homogeneous cytoplasm. Oenocytoids also contained phenoloxidase activity and were non-phagocytic. Thrombocytoids were reported to be rarest hemocyte type and were characterized as elongate cells (30 μm in length) with a homogeneous cytoplasm that were non-adhesive in vitro.

Using this classification scheme as a reference point, we determined that perfusion samples from *An. gambiae* and *Ae. aegypti* contained hemocytes matching the above descriptions for granulocytes, adipohemocytes and oenocytoids but we did not observe any cells that could unambiguously be identified as thrombocytoids (Fig. 2A). A small percentage of cells recovered from both species also did not morphologically correspond to any of the hemocyte types described above. These cells were 4–6 μm in diameter, spherical, non-adhesive, and had a high nuclear to cytoplasmic ratio as visualized by phalloidin and anti-H1 histone staining (see below). We classified these cells as prohemocytes, because of their resemblance to hemocytes named prohemocytes in other insects (Lackie, 1988; Lavine and Strand, 2002). Samples from the high injection/recovery method consisted primarily of granulocytes and much smaller percentages of prohemocytes and oenocytoids (Fig. 2B). However, almost no adipohemocytes were collected. The size and morphology of prohemocytes, granulocytes and oenocytoids collected from *An. gambiae* and *Ae. aegypti* using the high injection/recovery method were very similar to one another (Fig. 2C–H). The percentage, morphology and size of each hemocyte type in the proboscis and low injection/recovery samples was also very similar to the high injection/recovery samples (data not presented). Given the near absence of adipohemocytes in the proboscis and injection/recovery samples and their identical morphology to fat body cells in the hemocoel, we concluded that adipohemocytes were not hemocytes but rather contaminating fat body cells that were most frequently collected using the perfusion method.

Functional markers further discriminate hemocyte types

We next assessed whether any of the fluorescent or enzymatic markers listed in Table 1 discriminated the morphological hemocyte types described above using samples collected from sugar fed (non-immune challenged) 4 day old adult females. When first collected from mosquitoes, granulocytes were spherical or slightly tear-shaped cells that could not be fully distinguished from oenocytoids or prohemocytes by light microscopy alone. Unlike oenocytoids and prohemocytes, however, granulocytes rapidly bound and spread on the surface of glass slides (Fig. 3A, B). Staining with phalloidin and anti-H1 histone clearly visualized the filopodia, focal adhesions, and low nuclear/cytoplasmic ratio typical of granulocytes after spreading on glass slides (Fig. 3C). Phagocytosis assays further indicated that granulocytes

were the only hemocyte type in *Ae. aegypti* and *An. gambiae* that internalized bacteria (Fig. 3D–G).

Most granulocytes from both mosquito species spread symmetrically on glass slides but some cells spread asymmetrically with one axis clearly longer than the other (Fig. 3A–C). We originally thought these difference in spreading morphology could be functionally significant since other insects produce adhesive hemocyte types that assume different spreading morphologies in vitro which in turn correlate with different immune functions. In most Lepidoptera, for example, granulocytes spread symmetrically in vitro and are the professional phagocytes, whereas plasmatocytes spread asymmetrically, are non-phagocytic, and function as the main capsule-forming hemocyte type (summarized by Lavine and Strand, 2002; Strand et al., 2006). Analogously, the professional phagocytic hemocytes in *Drosophila* (called plasmatocytes) differ in size and assume a different spread morphology from lamellocytes that form capsules (Lanot et al., 2001; Irving et al., 2005). However, we found that symmetrically and asymmetrically spreading granulocytes from both mosquito species phagocytized bacteria. A small number of hemocytes also attached to glass fibers inserted into the hemocoel but did not form multilayered capsules typically observed in Lepidoptera or *Drosophila*. Inspection of these glass fibers indicated that both symmetric and asymmetrically spreading granulocytes were present.

Oenocytoids from *An. gambiae* and *Ae. aegypti* were distinguished from spread granulocytes by their spheroidal shape (6–20 µm diameter) and weak adhesion to glass or plastic (Fig. 4A). Oenocytoids were the only hemocyte type that stained positively for phenoloxidase activity and also were consistently labeled more strongly by MCB than granulocytes and prohemocytes (Fig. 4B–D). Oenocytoids from both species usually also had a single nucleus. The rounded morphology and lack of adhesion of prohemocytes was similar to oenocytoids. However, prohemocytes were usually smaller (4–6 µm) and had a larger nuclear to cytoplasmic ratio than oenocytoids (Fig. 4A). Prohemocytes also lacked phenoloxidase activity and were stained weakly or not at all by MCB. The other fluorescent probes and enzymatic assays we tested did not unambiguously discriminate a single hemocyte type. Diaminofluorescein diacetate and dihydrorhodamine 123 that detect reactive nitrogen and oxygen species respectively stained granulocytes and oenocytoids similarly but stained prohemocytes very weakly or not at all. The intracellular calcium marker Fluo3-FF-AM, wheat germ agglutinin, soybean lectin, and *Helix pomatia* lectin labeled all hemocytes with varying intensity as did assays for acid phosphatase activity.

We tested antibodies generated against six immune-related proteins from *An. gambiae* against hemocytes from sugar fed (non-immune challenged), 4 day old adult females of both mosquito species. Four of the antibodies (anti-PPO6, -SP22D, -SRPN6, -SRPN10) only stained hemocytes from *An. gambiae* while the remaining two (anti-PSMD3 and -LYS c-1) stained hemocytes from *An. gambiae* and *Ae. aegypti*. PPO6 is one of nine prophenoloxidases encoded by *An. gambiae* that is thought to be expressed predominantly, if not exclusively, in hemocytes and a subpopulation of cells in the 4a3b cell line that was derived from hemocytes (Muller et al., 1999; Christophides et al., 2002). Anti-PPO6, however, may cross-react with other PPOs (Muller et al., 1999). We found that anti-PPO6 stained only oenocytoids from non-immune challenged *An. gambiae* (Fig. 5A, B) which also were the only hemocytes that stained positively in phenoloxidase assays (see above). Previous in vivo studies detected Sp22D expression in unknown hemocytes present in circulation or bound to tissues like muscle and trachea (Danielli et al., 2000). We observed that anti-Sp22D strongly stained 20–25% of *An. gambiae* granulocytes but did not stain any oenocytoids or prohemocytes (data not presented). SRPN6 was previously detected in midgut epithelium and hemocytes of unknown type while SRPN10 was detected in the midgut, pericardial cells and hemocytes (Danielli et al., 2003; Abraham et al., 2005). We found that anti-SRPN6 and SRPN10 labeled the cytoplasm of both granulocytes

and oenocytoids in a punctate pattern but did not stain prohemocytes (data not presented). PSMD3 (formerly named DOXA2) is closely linked to a QTL for melanotic encapsulation of malaria ookinetes that is expressed in several tissues including hemocytes (Zheng et al., 2003; P. Romans, pers. commun.). Anti-PSMD3 weakly stained granulocytes and oenocytoids in *An. gambiae* (Fig. 5A, B). The same weak staining pattern was also observed in *Ae. aegypti* (data not presented). Lysozymes are a family of proteins produced by both invertebrates and vertebrates that are defined by their ability to cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl glucosamine in the peptidoglycan layer of bacterial cell walls. The activity of lysozyme c-1 from *An. gambiae* toward bacterial cell walls has not been determined but recent studies indicate that this protein is present in hemolymph and surprisingly reduces melanization of foreign targets (Li et al., 2005; Li and Paskewitz, 2006). Similar to anti-PSMD3, anti-LYS c-1 stained the cytoplasm of granulocytes and oenocytoids from *An. gambiae* and *Ae. aegypti* (data not presented).

Immune challenge affects labeling patterns of selected antibody markers

Although PPO6 is constitutively expressed in the 4a3b cell line (Mueller et al., 1999), immune challenge is well known to activate the PO cascade in adult mosquitoes (Jiang et al., 1997; Mueller et al., 1999). In contrast to sugar fed, non-immune challenged females, anti-PP06 stained both oenocytoids and granulocytes collected from *An. gambiae* females immune challenged with dead, FITC-labeled *E. coli*. This is illustrated by comparing the confocal images presented in Fig. 5A–D which were obtained using identical settings for gain, aperture, and laser intensity. Phenoloxidase assays produced the same pattern (data not presented) suggesting that PPO6 and possibly other PPOs are constitutively expressed in oenocytoids but are upregulated in granulocytes after exposure to bacteria. Immune challenge with *E. coli* resulted in staining of all granulocytes by anti-SP22D (data not presented). Compared to samples from non-challenged mosquitoes (Fig. 5E, F), we also observed an increase in staining intensity of granulocytes and oenocytoids following immune challenge using anti-PSMD3 (Fig. 5G, H). No changes in staining patterns after immune challenge with *E. coli* were observed with anti-LYS c-1, -SPRN6, or -SRPN10 (data not presented). An overall summary of hemocyte staining properties using the functional assays, fluorescent probes and antibody markers we tested is presented in Table 3.

Adult males, pupae, and larvae contain the same hemocyte types as adult females

Most studies on hemocytes from *Drosophila* and Lepidoptera involve cells collected from larvae, whereas studies with mosquitoes focus on adult females since this is the life stage that vectors medically important pathogens. To assess whether the hemocyte types observed in adult females are present in other life stages, we collected hemocytes from 4 day old adult males, third stadium larvae, and 2 day old pupae using the high injection/recovery method. Hemocytes were easily collected from pupae and adult males, whereas the fragile nature of larvae made hemocyte collection more difficult. However, the use of fine glass needles for injections allowed us with practice to consistently collect large numbers of hemocytes from larvae of both species using the high injection/recovery method. T-tests indicated that fewer hemocytes on average were collected from *An. gambiae* adult males compared to adult females ($t=4.2$; $p<0.0001$) but no differences were found in the number of hemocytes recovered from adult male and female *Ae. aegypti* ($t=0.7$; $p>0.1$) (see Fig. 1A and 6A). Life stage comparisons indicated that significantly more hemocytes were collected from *An. gambiae* pupae and adult females than larvae and adult males ($F=5.1$; $p<0.001$). In contrast, no differences in the number of hemocytes recovered were found between life stages in *Ae. aegypti* ($F=0.7$; $p>0.1$). Classification of hemocyte types using the morphological and functional markers described above indicated that larvae, pupae and adult males contained similar percentages of granulocytes, oenocytoids and prohemocytes to four day old adult females (Fig. 6B–D). No other hemocyte types were observed.

Hemocyte abundance declines with adult age and transiently increases following a blood meal

The preceding studies focused on a single age class of larvae (third instar), pupae (three day) and adults (four day) in order to minimize possible age-related differences in hemocyte abundance when evaluating collection methods and markers for identification. Once these methods were standardized, we next asked whether hemocyte abundance changed in adult females with age or blood feeding since both greatly affect mosquito physiology (Raikhel et al., 2004). Hemocyte abundance progressively declined with age in both species when mosquitoes were fed only sugar water (Fig. 7A, B). In contrast, the number of hemocytes collected from the hemocoel transiently increased 24–48 h after a blood meal in comparison to sugar fed females of the same age (Fig. 7A, B). Hemocyte abundance then declined to the same levels as sugar-fed females by 72 h post-blood meal. Classification of the hemocyte types present 48 h after blood feeding did not reveal any differences in the percentage of granulocytes, oenocytoids and prohemocytes present compared to sugar fed females of the same age (data not presented).

Discussion

Most studies of mosquito hemocytes have relied exclusively on light or electron microscopy for identification (Andreadis and Hall, 1976; Foley, 1978; Kaaya and Ratcliffe, 1982; Drif and Brehelin, 1983). Studies in other insects, however, indicate that morphology alone is often inadequate for identification and that functional markers along with uniformity in collection and culture methods are needed to reliably distinguish one hemocyte type from another. Thus study, therefore, focused on three needs. First, we compared collection methods and examined different life stages in order to develop a comprehensive data set on the number and types of hemocytes present in mosquitoes. Second, we examined several functional markers to assess whether any could be used in combination with morphology to facilitate hemocyte identification. Some of the markers we tested have been used previously to classify hemocytes from *Ae. aegypti* (Hillyer and Christensen, 2002; Hillyer et al., 2003), whereas others had not. Third, we compared *An. gambiae*, whose hemocytes had not previously been classified, with *Ae. aegypti* to determine if similar criteria can be used in both.

We conclude that hemolymph from *An. gambiae* and *Ae. aegypti* contains three hemocyte types that are present in larvae, pupae and adults. Granulocytes are by far the most abundant cell type while oenocytoids and prohemocytes together usually comprise less than 10% of the total hemocyte population. Our results also indicate that collection method greatly affects the number and types of cells obtained when bleeding mosquitoes. The high injection/recovery method yielded the largest number of hemocytes per individual and produced lower levels of contaminants than the perfusion method. This improvement appears to be due to the use of an anticoagulant and a pipette to collect the diluted hemolymph more cleanly. The use of Schneider's medium also improved hemocyte viability in primary culture compared to other methods we tested. The importance of reducing contamination cannot be overemphasized because its presence makes identification and accurate counting of mosquito hemocytes very difficult. For example, adult *Ae. aegypti* were previously reported to contain 2000 hemocytes (Christensen et al., 1989), whereas subsequent studies suggested this was an overestimate because of fat body and other cellular contaminants in perfusates (Hillyer and Christensen, 2002). We too found it difficult to accurately discriminate hemocytes from other contaminants in perfusion samples. The presence of contaminants also affects hemocyte morphology and spreading behavior which further exacerbates cell identification. In particular, granulocytes phagocytize contaminants which reduces binding of these cells to glass slides and alters spreading morphology. Reducing contamination from other cells and tissues like the fat body is also of obvious importance in any functional genomic study that seeks to identify genes

preferentially expressed in hemocytes or a specific hemocyte type (Bartholomy et al., 2004; Irving et al., 2005).

The most common types of hemocytes reported in the literature are prohemocytes, granular cells (=granulocytes), plasmatocytes, spherule cells and oenocytoids. These hemocyte types have been described from species in diverse orders including Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (see Jones, 1962; Lackie, 1988; Lavine and Strand, 2002; Ribeiro and Brehelin, 2006). As noted in the introduction of this paper, these hemocyte names have also been used in prior studies to classify the types of hemocytes observed in mosquitoes. In insects like Lepidoptera, granulocytes are usually the professional phagocytes, plasmatocytes are the main capsule forming cell, oenocytoids are a source of phenoloxidasases, prohemocytes are putative stem cells, and spherule cells are potentially a source of cuticular components. In contrast, the most detailed data on hematopoiesis in insects derives from *Drosophila* whose hemocytes, for historic reasons, are named differently from most other insect species. *Drosophila* larvae contain three recognized types of hemocytes in circulation named plasmatocytes, lamellocytes and crystal cells (Lanot et al., 2001). While rare, hemocytes similar to prohemocytes in other insects, are also observed in circulation in *Drosophila*, whereas no hemocytes resembling spherule cells are observed (Lanot et al., 2001). Plasmatocytes are the professional phagocyte, lamellocytes are specialized capsule forming cells, and crystal cells are a primary source of phenoloxidase activity. Based on morphology and functional activity *Drosophila* plasmatocytes are most analogous to hemocytes named granulocytes in Lepidoptera and other insects, lamellocytes are most similar to plasmatocytes, and crystal cells are analogous to oenocytoids (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006). With this background in mind, we carefully considered whether to name the hemocytes observed during the current study using the terminology of previous studies on mosquitoes or to adopt to the terminology used for *Drosophila*. In the end, we decided to continue using the nomenclature adopted in previous studies on mosquito hemocytes, because it was less confusing and made it easier to compare our data to earlier results. There clearly is a need though for workers studying hemocytes from mosquitoes, *Drosophila*, Lepidoptera, and other insects to develop a more uniform terminology.

Although some authors have named phagocytic, adhesive hemocytes from mosquitoes as plasmatocytes (Drif and Brehelin, 1983; Kaaya and Ratcliffe, 1982), we concur with Hillyer and Christensen (2002) in naming these cells granulocytes since they most closely conform to the characteristics of hemocytes named granulocytes in most other insects (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006; see above). Our functional bioassays and markers also did not indicate that mosquitoes produce more than one adhesive type of hemocyte even though lepidopterans (granulocytes and plasmatocytes) and *Drosophila* (plasmatocytes and lamellocytes) produce two adhesive hemocyte types specialized for phagocytosis and capsule formation respectively (Gillespie et al., 1997; Lavine and Strand, 2002; Lebetzky et al., 2000; Irving et al., 2005; Strand et al., 2006). Mosquito oenocytoids are identified by the combination of morphology, MCB labeling, and phenoloxidase activity. The correlation between elevated MCB labeling and phenoloxidase activity is potentially important because MCB reacts with glutathione that is a well known inhibitor of melanization (Pech et al., 1994). Since insect PPOs lack signal peptides, they are thought to be stored in the cytoplasm and released when activated oenocytoids lyse following immune challenge (Pech et al., 1994; Jiang et al., 1997; Cho et al., 1998; Huang et al., 2001). Elevated levels of intracellular glutathione thus may play a role in blocking PPO activation in oenocytoids prior to lysis. Phenoloxidase activity was previously reported to be oenocytoid specific in *Ae. aegypti* (Hillyer and Christensen, 2002), but phenoloxidase activity was detected in putative granulocytes from *Anopheles albimanus* (Hernandez et al., 1999). Our results indicate that oenocytoids constitutively exhibit phenoloxidase activity, whereas granulocytes exhibit increased phenoloxidase activity following immune challenge. This suggests that regulation

of the phenoloxdase cascade potentially differs between these hemocyte types. Differences in Sp22D expression following immune challenge suggests that functionally distinct subpopulations of granulocytes may also exist.

The term prohemocyte has historically been used for putative hemocyte progenitor cells with the capacity to differentiate into other cell types (Jones, 1970; Lackie, 1988; Lavine and Strand, 2002). Kaaya and Ratcliffe (1982) concluded that mosquito hemolymph contains prohemocytes, whereas Hillyer and Christensen (2002) suggested these objects were more likely subcellular debris. We also observed contaminants in perfusion samples that could be mistaken for small cells. However, samples collected by probocis clipping and injection/recovery convince us that *An. gambiae* and *Ae. aegypti* hemolymph contains a hemocyte type that is morphologically and functionally distinct from granulocytes and oenocytoids. The uniform size, rounded morphology, large nuclear to cytoplasmic ratio, and lack of labeling of these cells by the functional markers we tested are consistent with these cells being a type of progenitor cell. However, it is possible these cells could be more specialized hemocyte type, such as a granulocyte precursor rather than a stem cell capable of differentiating into either granulocytes or oenocytoids. Additional developmental and functional studies will be needed to understand the lineage fate and activity of each hemocyte type present in *An. gambiae* and *Ae. aegypti*.

Our results indicate that the hemocyte types observed in adult females are also present in adult males, pupae, and larvae of both mosquito species. The number of hemocytes collected from adult, sugar-fed mosquitoes also progressively declines with age which has also been implicated in increased susceptibility to septic infection in *Ae. aegypti* (Hillyer et al., 2005). However, blood feeding transiently increased the number of hemocytes we were able to collect. The underlying mechanism for this effect or whether other perturbations, like injection of bacteria, also increase hemocyte numbers is currently unknown. One possibility is that blood feeding transiently increases the number of hemocytes that are in circulation versus sedentary on tissues. Another is that blood feeding stimulates increased proliferation of one or more hemocyte types.

In summary, our results lay a foundation for collecting and identifying hemocytes from two mosquito species using light and fluorescence microscopy methods. These approaches are also fully compatible with fluorescence-activated cell sorting (FACS) methods that have proven essential for functional studies of mammalian immune cells. With development of additional markers and sorting methods, studies on lineage relationships, hematopoiesis, and the responses of specific genes in different hemocyte populations following immune challenge should be feasible in mosquitoes.

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Fig. 1.

Collection method affects hemocyte number and the level of contaminants recovered from *An. gambiae* and *Ae. aegypti* adult females. A. Total hemocyte counts $\pm$ SE recovered per mosquito by perfusion, clipping the proboscis, low injection/recovery, and high injection/recovery. A minimum of 10 individuals were bled per treatment ($F_{7,77} = 34.7$; $p < 0.0001$). Means with the same letter do not significantly differ from one another (Tukey-Kramer HSD multiple comparison procedure $\alpha = 0.05$). Low magnification differential interference contrast (DIC) micrographs of samples collected by perfusion (B) or high injection/recovery (C). The perfusion sample contains large amounts of contaminants along with hemocytes while the injection/recovery sample contains primarily hemocytes with low levels of contamination (see

text). Representative granulocytes (Gr) and prohemocytes (Pr) are indicated. Scale bar = 40 μm .

Fig. 2.

Differential hemocyte counts for perfusion (A) and high injection/recovery (B) samples from *An. gambiae* and *Ae. aegypti*. Counts were made on the same samples used to produce the total hemocyte counts presented in Fig. 1. Hemocytes were classified as granulocytes, oenocytoids, prohemocytes, and adipohemocytes on the basis of morphology and staining with different markers (see text). DIC images of a representative prohemocyte (C), granulocyte (D) and oenocytoid (E) from an *An. gambiae* adult female. A prohemocyte (F), granulocyte (G), and oenocytoid (H) from an *Ae. aegypti* adult female is also shown for comparison. Note the close similarity in morphology and size of each hemocyte type from the two species. Scale bar = 25 μ m.

Fig. 3. Granulocytes from *An. gambiae* and *Ae. aegypti* adult females. (A, B) DIC images of granulocytes from *An. gambiae* 1 h after placement on a glass slide in Schneider's medium plus 10% FBS. One cell (A) has spread asymmetrically while the other (B) has spread symmetrically. Granules (G) are visible in the granulocyte presented in B. (C) Confocal image of *An. gambiae* granulocytes stained with phalloidin that labels F-actin (green) and anti-histone H1 that labels nuclei (red). Filopodia (Fp) and focal adhesions (Fa) associated with F-actin and adhesion to the glass slide are clearly visible. The projected composite image was generated from 6 optical sections that were one half micron in thickness. Scale bar in C = 10 μ m with the same magnification in A and B. Low magnification DIC (D) and epifluorescent image (E) of

hemocytes from *An. gambiae* 1 h after incubation with FITC-labeled *E. coli*. DIC (F) and epifluorescent (G) images of hemocytes from *Ae. aegypti* 1 h after incubation with FITC-labeled *E. coli* is also presented. All granulocytes (Gr) from both species phagocytized bacteria (green), whereas oenocytoids (Oe) and prohemocytes (Pr) have not. Scale bar in G= 80 μ m with the same magnification in D–F.

Fig. 4.

(A) Confocal image of *Ae. aegypti* hemocytes stained with phalloidin (green) and anti-histone-H1 (red). Spread granulocytes (Gr) are distinguished from round, unspread oenocytoids (Oe) and smaller, unspread prohemocytes (Pr). Also note the visibly higher nuclear to cytoplasmic ratio in the prohemocyte compared to the oenocytoids. Scale bar= 20 μ m. The projected composite image was generated from 6 optical sections that were one half micron in thickness. (B) Higher magnification DIC image of an oenocytoid. A DIC image of an oenocytoid following staining for phenoloxidase activity (C), and an epifluorescent image of an oenocytoid following vital staining with monochlorobimane (MCB) (D) are also shown. Staining of An.

gambiae oenocytoids for phenoloxidase activity and MCB is virtually identical to the images shown for *Ae. aegypti*. Scale bar = 15 μ m in B with the same magnification in C and D.

Fig. 5. Bacterial challenge alters staining of *An. gambiae* hemocytes using antibodies to PPO6 and PSMD3 (formerly Dox-A2). DIC (A) and confocal (B) micrographs of hemocytes from non-immune challenged mosquitoes stained with anti-PPO6 and a Texas-red conjugated secondary antibody (1:1000). The two oenocytoids in the image are stained strongly whereas the granulocyte is stained weakly. DIC (C) and confocal (D) micrographs of hemocytes collected 3 h post-injection of FITC-conjugated *E. coli*. Hemocytes were stained with same concentration of PPO6 antiserum and secondary antibody as in A and B. Note that granulocytes are stained more strongly in the challenged sample compared to the non-challenged sample. In contrast, staining intensity of the oenocytoids in the challenged and non-challenged samples are similar.

The projected composite images in B and D were generated from 4 optical sections that were one half micron in thickness. Laser intensity, gain, and aperture settings in acquiring the two images were also identical. (E and F) DIC and epifluorescent micrographs of hemocytes from non-immune challenged *An. gambiae* hemocytes stained with anti-PSMD3 and a FITC-conjugated secondary antibody. All cells in the image are uniformly but weakly labeled. (G and H) DIC and epifluorescent micrographs of hemocytes collected 3 h post-injection of rhodamine-conjugated *E. coli*. Note the stronger staining of granulocytes (Gr) compared to the non-challenged sample. In contrast, PSMD3 staining of the prohemocyte in the image remains weak. Hemocytes were stained with same concentration of anti-PSMD3 and secondary antibody as in E and F. Camera exposure time and gain settings in acquiring the images in F and H were identical. Scale bar in H= 40 μm with the same magnification in E–G.

Fig. 6. Hemocyte counts from different life stages of *An. gambiae* and *Ae. aegypti* larvae. (A) Total hemocyte counts from larvae (third instar), pupae (day 4) and adult males (day 4). (B–D) Differential hemocyte counts from larvae, pupae, and adult males respectively. Hemocytes were collected from each stage using the high injection/recovery method. A minimum of 10 individuals were sampled for each life stage. Hemocytes were placed on glass slides in Schneider's medium plus 10% FBS for 1 h and then identified by morphology, monochlorobimane, and rhodamine 123 staining. See Fig. 1A and Fig. 2 for total and differential hemocyte counts from adult females.

Fig. 7. Total hemocyte counts from adult female *An. gambiae* (A) and *Ae. aegypti* (B) of increasing age (days). Cohorts of females fed sugar only were sampled daily using the high injection/recovery method. A second cohort of females from the same starting population were blood fed on day 3 and thereafter sampled on days 4, 5 and 6. Asterisks above the bar indicate that the total number of hemocytes collected from blood fed females differed significantly from females of the same age that were fed sugar water only (t-test, $\alpha = 0.05$). A minimum of 10 individuals were bled per time point in each treatment.

Table 1

Non-antibody probes tested as markers for mosquito hemocytes.

Reagent	Source	Concentration	Reactivity
Monochlorobimane	Molecular probes	10 μ M	Intracellular glutathione
Diaminofluorescein diacetate	Sigma	10 μ M	Intracellular nitric oxide and Peroxynitrite
Rhodamine 123	Sigma	20 ng/ml	Intracellular reactive oxygen
Fluo3-FF-AM	Sigma	0.3 μ M	Intracellular calcium
Propidium iodide	Santa Cruz Biotechnology	2.0 μ g/ml	Cell-impermeable nucleic acid dye (vital dye)
Alexa 488 phalloidin	Molecular Probes	1: 200 dilution	F-actin
3,4 Dihydroxy- -phenylamine	Sigma	2 mg/ml	Intracellular Phenyloxidase activity
Glycerol 2- phosphate	Sigma	1 mM	Intracellular acid phosphatase
FITC-conjugated <i>Escherisia coli</i>	Lab stock	1 x 10 ³ cells mosquito	Phagocytosis
FITC-conjugated peanut lectin	Sigma	10 μ g/ml	β -gal(1–3) Galactosamine
FITC-conjugated soybean lectin	Sigma	10 μ g/ml	Surface α/β -N-acetyl Galactosamine and galactopyranoside
FITC-conjugated wheat germ lectin	Sigma	10 μ g/ml	Surface N-acetyl Glucosamine and N-acetyl neuramine acid

Table 2

Antibody probes tested as markers for mosquito hemocytes.

Antisera	Dilution	Antigen	Source
anti-H1 histone	1: 200	Cell nuclei	Santa Cruz Biotech.
anti-SRPN6	1: 1000	Serpin6 <i>An. gambiae</i>	Abraham et al., (2005)
anti-SRPN10	1: 100	Serpin10 <i>An. gambiae</i>	Danielli et al., (2003)
anti-PPO6	1: 500	Phenoloxidase 6 <i>An. gambiae</i>	Mueller et al., (1999)
anti-SP22D	1: 500	Chitin binding serine protease SP22D <i>An. gambiae</i>	Danielli et al., (2000)
anti-PSMD3	1: 800	Regulatory subunit 26S proteasome <i>An. gambiae</i>	Romans, unpubl.
anti-LYS c-1	1: 200	Lysozyme-c1 <i>An. gambiae</i>	Li and Paskewitz, (2006)

Table 3
Summary of hemocyte staining patterns in *An. gambiae* and *Ae. aegypti* adult females.

	<i>An. gambiae</i> [*]			<i>Ae. aegypti</i> [*]		
	Gr	Oe	Pr	Gr	Oe	Pr
General Markers						
Adhesion to foreign surfaces	+	-	-	+	-	-
Monochlorobimane [†]	+	+	-	+	+	-
Phagocytosis of FITC-labeled <i>E. coli</i>	+	-	-	+	-	-
Phenoloxidase activity ^{††}	+	+	-	+	+	-
Diaminofluorescein diacetate	+	+	-	+	+	-
Dihydrothodamine 123	+	+	-	+	+	-
Fluo3-FF-AM	+	+	+	+	+	+
Peanut lectin	+	+	+	+	+	+
Soybean lectin	+	+	+	+	+	+
Wheat germ lectin	+	+	+	+	+	+
Acid phosphatase	+	+	+	+	+	+
Antibody markers						
anti-SRPN6	+	+	-	-	-	-
anti-SRPN10	+	+	-	-	-	-
anti-PPO6 ^{††}	+	+	-	-	-	-
anti-SP22D ^{††}	+	-	-	-	-	-
anti-PSMD3 ^{††}	+	+	-	+	+	-
anti-LYS c-1	+	+	-	+	+	-

* Gr= granulocyte, Oe= Oenocytoid, Pr= Prohemocyte.

[†] Monochlorobimane stains oenocytoids from both mosquito species more strongly than granulocytes while prohemocytes are stained weakly or not at all (see text).

^{††} Staining for phenoloxidase activity and PPO6 was restricted almost exclusively to oenocytoids in non-immune challenged mosquitoes but granulocytes also exhibit phenoloxidase activity and PPO6 staining following immune challenge by bacteria (see text). Differences in staining patterns also exist for anti-SP22D and anti-PSMD3 between non-challenged and immune-challenged mosquitoes (see text).